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On the N-terminal amino acids in fibrinogen and fibrin

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With 2 figures in the text

Introduction

As early as 1872 Alexander Schmidt characterized thrombin as an enzyme. The hypothesis that proteolysis was involved in the conversion of fibrinogen to fibrin was shortly afterwards proposed by several authors, although this concept was found to be based on inconclusive evidence. The resemblance of normal coagulation to that caused by enzymes like trypsin, papain and enzymes from certain snake venoms made Eagle and Harris in 1937 suggest proteolysis as a factor of importance in blood coagulation. Direct evidence of such proteolysis was not obtained until 1951, when Bailey, Bettelheim, Lorand & Middlebrook, using Sanger's technique, showed a difference between the N-terminal amino acids of bovine fibrinogen and fibrin. Thus in fibrin, glycyl residues replaced the glutamyl residues in the fibrinogen, and the glutamyl residues were found in the clot supernatant, suggesting a proteolytic splitting off of glutamyl peptides during the conversion of fibrinogen to fibrin. Peptide material containing N-terminal glutamic acid was also isolated from the clot supernatant by these authors (Bettelheim & Bailey, 1952; Lorand, 1952; Bailey & Bettelheim, 1955; Bettelheim, 1956). An excellent review of this earlier work on the transformation of fibrinogen to fibrin was recently given by Bailey & Bettelheim (1955).

Some uncertainty seems to exist regarding the number of N-terminal amino acids per unit weight of fibrinogen and fibrin, due to difficulties in the quantitative analysis of N-terminal amino acids by the DNP (dinitrophenyl) method.

Lorand & Middlebrook (1952) found, after correction for destruction, one N-terminal glutamyl and two N-terminal tyrosyl residues in a 450,000 unit weight of bovine fibrinogen. In the corresponding amount of fibrin they found two tyrosyl and four glycyl end-group residues. Bailey & Bettelheim (1955), assuming a molecular weight of 330,000 for bovine fibrinogen, considered the most probable number of N-terminal residues to be one glutamyl and two tyrosyl residues per mole of fibrinogen, and two tyrosyl and two or three glycyl residues in an approximately equal weight of fibrin.

Species differences of fibrinogen were revealed by end-group studies, when Lorand & Middlebrook (1953) showed that human fibrinogen contained N-terminal alanyl residues instead of the glutamyl residues present in bovine fibrinogen.

When a highly purified fibrinogen recently became available in this laboratory (Blombäck & Blombäck, 1956), a reinvestigation of the N-terminal amino acids in bovine fibrinogen and fibrin was undertaken by using Edman's phenylthiocarbamyl

method. In addition, fibrinogen and fibrin from different species were investigated, as well as fibrin formed by the action of papain and a snake-venom enzyme (Reptilase). A short communication covering part of this work has been published recently (Blombäck & Yamashina, 1957).

Materials and methods

Fibrinogen

The method for preparation of *human and bovine* fibrinogen with a coagulability of 98–100 % has been published elsewhere (Blombäck & Blombäck, 1956). The coagulability of the bovine fibrinogen was usually 99 %, judged from the spectrophotometric assays, whereas that of the human material was about 1 % lower.

Pig fibrinogen was prepared in the same way as the bovine material. The coagulability of the fibrinogen was about 98 %. It can be noted that very large amounts of cold-insoluble globulin complexes were present in pig plasma and in fraction I.

Sheep, goat and dog fibrinogen were most conveniently prepared in the same manner as bovine fibrinogen. The sheep and dog fibrinogens were coagulable with thrombin to 92–95 %, and goat fibrinogen to about 90 %.

In preparing *horse fibrinogen* with the glycine method, some difficulty arose in obtaining satisfactory purification. A modification of the original method for human fibrinogen was therefore elaborated. Fraction I-2 was prepared in the usual way. In order to ensure quantitative removal of some globulin contaminants, fraction I-3 had to be precipitated at an ethanol concentration of 2 %, instead of 0.75 % as used in the original procedure. The high solubility of this globulin complex in horse plasma is remarkable. The fraction had a yellowish colour. After removal of fraction I-3 by centrifugation, the fibrinogen was precipitated by increasing the ethanol concentration to 6.5 %. The coagulability of the fibrinogen was 94 %.

Since glycine had been used in the preparation of fibrinogen, it had to be removed by dialysis before coupling the fibrinogen with phenylisothiocyanate. After 20–30 hours' dialysis in the cold against 0.3 *M* NaCl, which was changed at least three times, only traces of free glycine were discernible in the fibrinogen solution, but dialysis for 48 hours was needed to remove all traces of glycine. The fibrinogen was usually dialyzed for only 20–30 hours, as it was observed that an appreciable decrease in the coagulability of the fibrinogen took place during prolonged dialysis. However, even then the coagulability of the protein always decreased by 1–3%, probably due to some denaturation. By passing the dialyzed bovine fibrinogen solution (adjusted to pH 9.0 with 0.3 *M* tris (hydroxymethyl) aminomethane buffer) over a Dowex-2 × 8 (200–400 mesh) column in acetate form, buffered at pH 9.0 with a tris-acetate buffer, ionic strength 0.15, the coagulability of the effluent fibrinogen solution was restored to the original value. It is supposed that the denaturated protein is adsorbed on the ion exchanger. This procedure was, however, usually omitted, since dialysis produced no change in the N-terminal amino acids.

Thrombin (bovine)

Prothrombin was prepared according to Seegers, Loomis & Vandenbelt (1945). In order to get a higher yield of conversion with thromboplastin and calcium, the last purification steps following the elution from $Mg(OH)_2$ were omitted. After dialysis, the prothrombin was converted to thrombin by means of calcium and brain thromboplastin (prepared according to Owren, 1949),

dialyzed and lyophilized. Further purification of the thrombin was achieved by fractionation with methanol. The crude thrombin was dissolved to a protein concentration of about 1 % in 0.09 *M* acetate buffer, pH 5.9, containing 0.014 *M* calcium, and the solution cooled to 0°C. A 50 % methanol-water mixture was slowly added to a final methanol concentration of 10 %. During the addition, the temperature was lowered to − 3°C. The precipitate, consisting mainly of impurities derived from the thromboplastin preparation, was removed by centrifugation at the same temperature. The thrombin was then precipitated by adding slowly to the supernatant an equal volume of 50 % methanol to get an alcohol concentration of 30 %. The pH was at the same time lowered to 5.1 by adding dilute acetic acid. The precipitate was dissolved in water, dialyzed and freeze-dried. The activity was about 100–130 NIH[1] units per mg instead of about 30 NIH units per mg before fractionation. No fibrinolysis was induced with this preparation. Experiments were also performed with a highly active thrombin preparation containing 1380 NIH units per mg. It was obtained from highly purified prothrombin[2] by means of citrate activation, as described by Seegers, McClaughry & Fahey (1950).

Determination of protein

Fibrinogen and fibrin were determined spectrophotometrically after dissolving the protein in alkaline urea solution. The coagulability was determined as the ratio of coagulable protein to total protein in a fibrinogen preparation. In calculating the coagulability, the extinction coefficient ($E^{1\%}_{1\,cm}$) of fibrinogen was used for the coagulated sample as well.

Bovine thrombin was, when not otherwise stated, always used for the conversion of fibrinogen to fibrin. Coagulation was performed at pH 6.35 ± 0.05, ionic strength 0.15, and a protein concentration of 0.5–1.2 g per litre.

For further details of the determination procedures, see Blombäck (1958).

Preparation of fibrinogen and fibrin solutions for N-terminal amino acid determination

The dialyzed fibrinogen was diluted to about 1% with 0.3 *M* NaCl and used for coupling with phenylisothiocyanate. Fibrin was prepared by coagulation of fibrinogen under the standard conditions just described, but on a larger scale, i.e., 100–150 mg of protein in each beaker. The clot was synerized, washed thoroughly with 0.15 *M* NaCl and then dissolved in a solution of 40% urea in 0.15 *M* tris buffer, pH 9.0–9.5, during 2–4 hours, to a fibrin concentration of about 1%.[3] After determination of the protein concentration, the solution was used for coupling.

Papain fibrin

The enzyme used in the present experiments was a crystalline product of Worthington, Freehold, New Jersey. The preparation, dissolved in a citrate buffer, was sufficiently active for the actual experiments without any activation with versene and cysteine.

The conditions for coagulation of fibrinogen with papain were the same as described above as regards pH and ionic strength, i.e., pH 6.35 ± 0.05, ionic strength 0.15.

[1] National Institutes of Health.

[2] Kindly supplied by Dr. S. Magnusson of this institute.

[3] Clots formed from plasma also dissolve easily in this solvent, if the disodium salt of ethylenediaminetetraacetic acid (1 mg/ml) is added to the plasma prior to coagulation (cf. Lorand, 1948).

The protein concentration was about 1.5 g per litre. A citrate buffer (0.025 *M*) was, however, used instead of the phosphate buffer, because of the low activity of papain in the latter. The fibrinogen solution was coagulated with 8 γ of papain per ml. Coagulation was performed at room temperature (20–22°C). Coagulation occurred fairly rapidly, but no true clot was formed. It appeared instead as a soft gel which, on stirring, formed a flocculent precipitate. After 3 hours the precipitate was collected by centrifugation, washed three times with 0.15 *M* NaCl, and immediately dissolved in 40% urea in 0.15 *M* tris buffer at pH 9.0 to a protein concentration of about 1%. The solution was subsequently used for coupling.

It was found that, under the conditions described, about 50% of the total fibrinogen could be converted to coagulated protein. The poor yield of fibrin is due to secondary proteolysis by papain. The peptides were removed by washing the clot with 0.15 *M* NaCl, as already mentioned.

Reptilase fibrin

Ampoules of 1 ml were obtained from Pentapharm A. G., Basel. The preparation contains the coagulating enzyme of the venom of *Bothrops jararaca*. Reptilase appears to exert only a thrombin-like activity in blood coagulation. The activity corresponded to 0.75 NIH units per ml. No fibrinolytic activity was ascertainable in the preparation (Blombäck, Blombäck & Nilsson, 1957).

Conversion of fibrinogen to fibrin with Reptilase was performed under the standard conditions described above, i.e., pH 6.35 $\pm$ 0.05, ionic strength 0.15, and a protein concentration of 1 g per litre.

Reptilase was added to a concentration corresponding to about 0.025 NIH units per ml. The mixture was left standing for 5–15 hours at room temperature (20–22°C). The clot was similar to that formed by thrombin under the same conditions. It was opaque and had a high tensile strength. The clot was collected by syneresis, dissolved in urea solution and used for coupling with phenylisothiocyanate. The yield of fibrin in the clot was about 85–90% of the initial fibrinogen.

N-terminal amino acid determinations

The N-terminal determinations were carried out according to the PTC method described by Edman (1950). In this method phenylthiohydantoins are formed from the N-terminal amino acids. The development of paper chromatographic methods by Sjöquist (1953) and Edman & Sjöquist (1956) for the identification and quantitative estimation of the phenylthiohydantoins makes the PTC method a simple and accurate tool in the field of N-terminal analysis. The PTC method has been adopted for N-terminal determination in various proteins by Edman and co-workers as well as by other authors (cf. Fraenkel-Conrat, Harris & Levy, 1955; Yamashina, 1956; Heirwegh & Edman, 1957).

1. *Phenylthiohydantoin derivatives of amino acids (PTH amino acids) as reference substances*

PTH amino acids were synthesized mainly according to the description of Edman (1950), except for threonine and glutamic acid. PTH threonine was synthesized by the method of Ingram (1953). PTH dehydrothreonine was obtained by refluxing

PTH threonine in 1 *N* HCl for 2 hours at 100°C. As to the glutamic acid, a considerable formation of PTH glutamic acid ethyl ester took place during crystallization of the thiohydantoin from an acidic ethanol–water mixture. The acidic condition was caused by the hydrochloric acid used for transformation of the phenylthiocarbamyl derivative (PTC) into the PTH derivative. The crystallization of the PTH glutamic acid could, however, conveniently be carried out from an acetone–water mixture. The ethyl ester as reference substance was obtained after complete esterification of PTH glutamic acid in 1 *N* hydrochloric acid containing 90% ethanol. The ethyl ester is clearly separable from the PTH glutamic acid on the paper chromatogram.

PTH cysteic acid and PTH glutamine were synthesized as described by Edman & Lauber (1956).

2. *Preparation of PTH amino acids from the amino ends of fibrinogen and fibrin*

Preparation of phenylthiocarbamyl derivatives (PTC)

An approximately 1% solution of fibrinogen or fibrin, prepared as stated above, was mixed with an equal volume of pyridine. In most cases, 10 ml of the protein solution was used. To this mixture, phenylisothiocyanate was added, the amount being 25 μl per ml of mixture. Coupling was carried out for 2 hours at room temperature, with occasional addition of 1 *N* NaOH to keep the pH at 8.5–9.0. Tris buffer could be used for this purpose, provided that the PTC protein in the following procedure was precipitated with acetone. The buffer was added to the coupling mixture to give a final concentration of about 0.03 *M* and a pH of 9. During the subsequent treatment of the PTC protein by acetone, phenylisothiocyanate derivatives of tris were removed, which otherwise would have produced spots on the paper chromatogram.

After coupling, the reaction mixture was washed several times with an equal volume of benzene. Benzene readily extracts pyridine and the excess of phenylisothiocyanate, but even after extraction by benzene with vigorous mechanical stirring, traces of side-reaction products of phenylisothiocyanate often remained in the aqueous phase.

After washing the coupling mixture with benzene, the PTC protein in the aqueous phase was precipitated with 4 volumes of acetone, and the precipitate washed twice in the centrifuge tube with aqueous acetone. The precipitate was first washed with acetone for a few minutes, whereupon 1/5 volume of water was added and the washing continued for some minutes. This treatment has been found to remove interfering impurities formed during coupling. When the determination included peptides or amino acids, the aqueous phase containing PTC peptides or PTC amino acids was lyophilized directly after the washings with benzene, since the PTC peptides and the PTC amino acids are soluble in aqueous acetone.

Cyclization

The lyophilized or desiccator-dried PTC derivatives were submitted to cleavage and cyclization. Cyclization in 1 *N* HCl at 100°C during 1 hour was chiefly applied. For the amount corresponding to 100 mg of fibrinogen, 8–10 ml of 1 *N* HCl was used. Cyclization in 1 *N* HCl at 100°C was practically complete after 15 min., and no destruction of the PTH amino acids derived from the protein occurred even after 1 hour's cyclization (Table 4). PTH glutamine in bovine fibrinogen could, however,

be detected only by adopting a mild method of cyclization, namely, pH 2 at room temperature for 24–48 hours. Even then, the yield was fairly low.

The PTH amino acids were extracted from the cyclization mixture with an equal volume of ethyl acetate. Extraction was repeated three times and the combined extracts washed with water. The washed extract was evaporated to dryness at room temperature by means of a jet of air. The final residue was dissolved in a mixture of ethyl acetate and acetone (8:2 by volume) in glass tubes (11 × 70 mm) with ground-in stoppers. The volume of the solvent was chosen so that the concentration of each PTH amino acid did not exceed 1.5 μg per μl. Thus the PTH amino acids formed from 100 mg of fibrinogen were dissolved in 100–150μl of ethyl acetate–acetone solution. After complete solution of the PTH amino acids, about 10 μl of the solution was used for each chromatographic run.

Ethyl acetate, ether or ethylene chloride have often been used by several workers as solvents suitable for giving relatively high concentrations of the PTH amino acids. However, PTH amino acids do not go into solution in these solvents as quickly as expected, especially in the presence of various kinds of impurities formed during the procedure of N-terminal amino acid determination on proteins. A mixture of ethyl acetate and acetone, as mentioned above, was found to be the best solvent so far tested in this connexion. The PTH amino acids go into solution quantitatively if the mixture is left standing overnight at room temperature in a desiccator, saturated by the vapour of the same solvent. The samples can then be stored in the desiccator at +4°C for at most two weeks. Due attention must be paid to the risk of evaporation of the solvent. Thus, when samples are taken from the tubes these are always kept immersed in ice-water.

Since PTH histidine and PTH arginine are not extracted to any appreciable extent by ethyl acetate from an acidic aqueous solution, the aqueous phase after extraction with ethyl acetate was evaporated to dryness *in vacuo*, and the residue repeatedly extracted with water. The water extract was evaporated again, and the final residue dissolved in a small amount of water for the subsequent chromatography.

As cysteine is decomposed during the N-terminal determination procedure, an attempt was made to demonstrate cysteine in ox fibrinogen, after oxidation of cysteine to cysteic acid with performic acid. Oxidation was carried out according to Hirs (1956) at −10°C. After lyophilization, the oxidized fibrinogen was dissolved in 40% urea in 0.15 *M* tris buffer, pH 9.0–9.5, and submitted to coupling and the usual cyclization. The PTH amino acids derived from the oxidized fibrinogen were extracted first with ethyl acetate, and the aqueous phase lyophilized. The dry residue was then repeatedly extracted with 70% aqueous acetone (Edman & Lauber, 1956). The acetone extract was evaporated and the residue dissolved in a small volume of water for subsequent chromatography and electrophoresis.

3. *Paper chromatography*

Paper chromatographic techniques were used for identification and quantitative estimation of PTH amino acids. Sjöquist (1953) first described paper chromatography of PTH amino acids and later, together with Edman, developed the technique for quantitative purposes (Edman & Sjöquist, 1956). Among the solvent systems tried, solvents D, E and F of Edman & Sjöquist were most frequently used. We have found two additional solvents, G and H, to be of value. Solvent G is a modification of solvent F, and solvent H is a combination of solvents D and E.

With solvent H, the PTH of threonine and dehydrothreonine could be determined separately. Solvent G permitted determination of PTH glutamic acid ethyl ester. The R_f values of PTH amino acids in these two solvents are listed in Table 2, where the composition of the solvents is also described.

Particular precautions had to be taken when chromatography was carried out with solvents F and G. Two hours were required for getting equilibrium between the paper and the lower phase of the solvents in the chromatography jar. Solvents D, E and H were, on the contrary, very easy to handle and no equilibration prior to chromatography was necessary. The paper used was Whatman No. 1.

In the present studies, glycine, alanine and tyrosine could be determined with any of the solvents F, E, H and D. Dehydrothreonine was most conveniently determined in solvent H. The PTH of glutamic acid and glutamic acid ethyl ester could be determined only with solvents F and G, as these solvent systems contain no formamide. PTH derivatives of both glutamic acid and its ester were destroyed by formamide during drying of the paper after chromatography. Destruction occurred even at room temperature. It seems probable that PTH aspartic acid will behave in the same manner as PTH glutamic acid. It has been observed that the yields of PTH glycine and PTH tyrosine obtained with solvent E are fairly dependent on the purity of the solvents as well as on the paper used. Careful chromatography with solvent F has proved to give the most reproducible results with regard to these PTH amino acids.

Separation of PTH cysteic acid was performed by means of paper electrophoresis at pH 4.1 in 0.05 *M* acetate buffer (Edman & Sjöquist, 1956; Heirwegh & Edman, 1957), or by paper chromatography according to Titani, Yoshikawa & Satake (1956). Prior to chromatography, the paper was treated with 0.1 *M* citrate buffer, pH 4.2, saturated with *n*-butanol. Whatman No. 1 paper was used for chromatography as well as for electrophoresis.

After chromatography, the paper was dried at about 80°C for 20–30 minutes. The position of the PTH amino acids on the paper was marked with the aid of a fluorescence screen, as described by Edman & Sjöquist (1956). The marked spots were cut out, the strips put into tubes (11 × 70 mm) with ground-in stoppers and the PTH amino acids eluted with 2 ml of 95% ethanol at room temperature overnight. A paper blank of exactly the same size as the sample was cut out just beside the spot.

The ultraviolet absorption of the ethanol eluates was determined at 268 mμ for PTH glycine and alanine, at 323 mμ for PTH dehydrothreonine and at 270 mμ for the other PTH amino acids, using semimicro cells with a light-path of 1 cm. The extinction of a blank area of about 6.5 cm^2 was between 0.030–0.060. The extinction values of the samples should be 0.200–0.600. This extinction range corresponds to 5–15 μg of PTH alanine. At least three chromatographic runs were performed on every sample, with the sample in duplicate each time. The mean of all values was used. The standard deviation of a single value was 5–10% of the mean value. For calculation of the amount of the phenylthiohydantoins, the following molar extinction coefficients were used (see Fraenkel-Conrat, Harris & Levy, 1955); 14,500 for PTH glycine, 16,000 for PTH glutamic acid, 16,100 for PTH alanine, 16,400 for PTH threonine and PTH tyrosine and 25,700 for PTH dehydrothreonine (Δ-threonine).[1]

[1] These figures are for most PTH amino acids in good agreement with the recent data of Sjöquist (1957).

4. *Destruction of PTC and PTH amino acids during the determination procedure*

As mentioned above in connexion with the coupling procedure, there are some difficulties in removing side-reaction products solely by benzene extractions. Thus, a spot in solvent D with an R_f value of about 0.5 is often present when the washing with acetone has been omitted. Its R_f value is close to that of PTH dehydrothreonine. The ultraviolet absorption curve of this compound has a maximum at 275 mμ and a minimum around 245 mμ. In contrast to the PTH amino acids, it is decomposed very rapidly during the process of cyclization and is fairly weak after one hour (Table 1*C*). Impurities in the solvent front and the start are also frequently observed when the acetone washing has been omitted. Although the impurities do not result in any appreciable difficulties for determination on proteins, they seem to cause marked decomposition of the free PTC amino acids and, at any rate, of PTH tyrosine in the medium of 1 *N* HCl at 100°C. The destruction varies in the different amino acids. Thus, it is fairly slight in PTC glycine and pronounced in PTC tyrosine (Table 1*A*). PTC glutamic acid is quantitatively converted to an unknown derivative with a low R_f value (solvent F). Simul-

Table 1A. Over-all yield of amino acids in the absence of protein.

Amino acid	Added (μ moles)	Recovered (μ moles)	Yield (%)	Cyclization time (min.)
Glycine	2.0	1.601*	80.5	0
	2.0	1.524	76.2	15
	2.0	1.428	71.4	30
	2.0	1.335	66.8	45
Tyrosine	1.5	0.720*	48.0	0
	1.5	0.617	41.1	15
	1.5	0.515	34.3	30
	1.5	0.407	27.1	45

* Extrapolated value.

Table 1B. Paper chromatography (solvent E) of decomposition products formed during cyclization of PTC amino acids in 1 *N* HCl in the absence of protein.

(Bracketed figures denote the approximate R_f value.)

Amino acid	Extinction of PTH amino acid at 268 mμ	Extinction of derivative I at 270 mμ (0.6)	Extinction of derivative II at 270 mμ (0.9)	Cyclization time (min.)
Glycine	0.508	0.024	—	15
	0.476	0.036	—	30
	0.445	0.046	—	45
Tyrosine	0.233	0.062	0.015	15
	0.194	0.102	0.020	30
	0.153	0.125	0.034	45

Table 1C. Spots observed on paper chromatogram (solvent D).

In this experiment leucine was added to fibrinogen prior to the coupling. As can be seen, no decomposition of the PTC leucine took place during cyclization.

Amino acid added to the protein	Extinction of PTH amino acid at 270 mμ	Extinction of unknown spot at 270 mμ	Cyclization time (min.)
Leucine	0.314	0.700	10
	0.321	0.670	20
	0.312	0.216	40
	0.325	0.064	60

Table 2. R_f values of different PTH amino acids in solvents G and H.

Composition of the solvents.—Solvent G: 2 volumes of 75 % formic acid, 1 volume of ethylene chloride and 1 volume of *n*-heptane. The lower phase is applied to the paper lining of the chromatography jar, and the upper phase is put, after equilibration, into the trough. Solvent H: 1 volume of *n*-butyl acetate and 1 volume of xylene. This solvent is saturated with water and formamide. To 30 volumes of the saturated mixture is added 1 volume of propionic acid.

	Solv. H	Solv. G		Solv. H	Solv. G
Asparagine	0.03		Tryptophan	0.63	0.39
Aspartic acid	0.06	0.02	Δ-threonine	0.79	0.67
Glutamine	0.05	0.02	ε-PTC lysine	0.60	0.29
Glutamic acid	0.13	0.04	Valine	0.84	0.77
Glutamic acid ethyl ester		0.68	Leucine	0.91	0.83
Threonine	0.14	0.06	Isoleucine	0.89	0.84
Tyrosine	0.35	0.06	Phenylalanine	0.83	0.76
Glycine	0.37	0.22	Proline	0.84	0.80
Alanine	0.63	0.44			

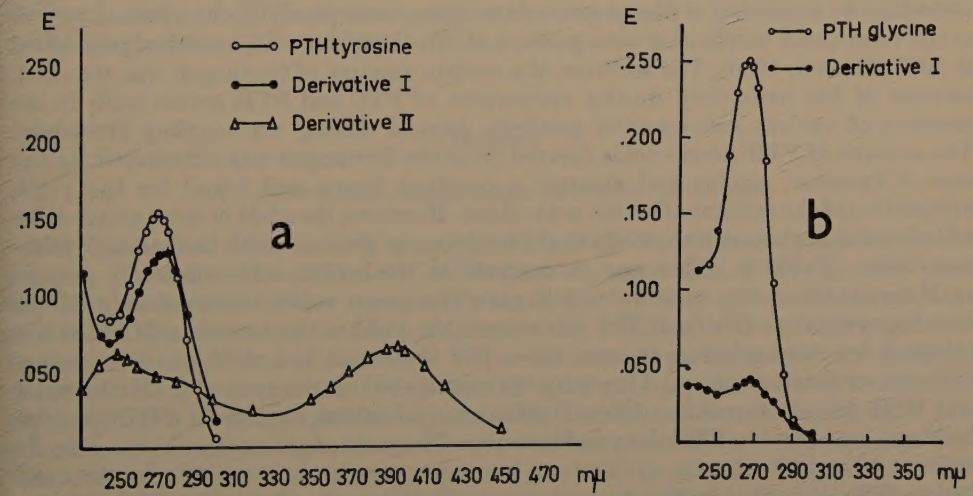

Fig. 1. (*a*) Absorption curves of PTH tyrosine and derivatives formed from PTC tyrosine in 1 *N* HCl at 100°C in the absence of protein. (*b*) Absorption curves of PTH glycine and derivative formed from PTC glycine in 1 *N* HCl at 100°C in the absence of protein.

taneously with the decreasing figures for the PTH amino acids, increasing amounts of decomposition products with varying R_f values appear (Table 1*B*). The ultraviolet spectra of the different compounds are recorded in Figs. 1*A* and 1*B*. Decomposition of the PTC amino acids is, however, slight or none if cyclization is performed in the presence of protein (Tables 1*C* and 3). Consequently, a trial to estimate the over-all yield of PTH amino acids formed from amino acids, under the same conditions as applied to fibrinogen and fibrin, would not be valid unless protein were present together with the amino acid. The estimation must be performed in a system containing the amino acid and a certain amount of protein, as will be described later.

5. *Yield of PTH amino acids in the whole procedure*

Differences in the conditions for cyclization of PTC derivatives to PTH amino acids can result in differences in yield, as recently pointed out by Edman (1956), on the basis of studies on the reaction mechanism of the transformation of PTC derivatives into PTH derivatives. In the present investigation, cyclization of the PTC derivatives has been performed in aqueous hydrochloric acid throughout. It then occurs rapidly, giving a good yield of the PTH amino acids.

As regards calculation of the yield of PTH amino acids, it has often been based on estimation of the ultraviolet absorption at 270 mμ, on the mixture of PTH amino acids prior to chromatographic separation (see Fraenkel-Conrat, Harris & Levy, 1955). In our experience, the yield of PTH amino acids could be estimated only on the ethanol eluate of PTH amino acids from the paper chromatogram. The values before chromatography are usually too high and not very reproducible. This depends on the extent to which the impurities have been removed by the washings (Tables 4 and 7).

Under the aforementioned experimental conditions involving cyclization in 1 *N* HCl at 100°C, estimation of the over-all yield of PTH amino acids from free amino acids was carried out as follows. A suitable amount of amino acid (about 1.5 μ moles) was added to a solution of fibrinogen, and coupling with phenylisothiocyanate as well as the subsequent treatments were performed. The coupling was, however, performed at 40°C (Edman, 1950). The addition of a certain amount of fibrinogen was required, because of the instability during cyclization of PTC and PTH amino acids in the presence of various side-reaction products formed during the coupling procedure. The amount of PTH amino acids derived from the fibrinogen was subtracted. In the case of tyrosine, glycine and alanine, a constant figure was found for the yield, irrespective of the amount of amino acid added. However, the yield of most amino acids added to the protein still showed a slight tendency to decrease with increasing cyclization times (Table 3). This was in contrast to the amino acids originally present as N-terminals in the protein, which gave the same value independently of the time for cyclization (Table 4). For this reason, the yield of the amino acids added was obtained by extrapolation to zero time. For threonine, the yield was the same at different cyclization times. Threonine was estimated as the sum of PTH threonine and PTH dehydrothreonine. After 60 minutes' cyclization, almost all PTH threonine had been converted to PTH dehydrothreonine. The yield of glutamic acid was also the same at 40 and 60 minutes' cyclization, but was somewhat lower at 20 minutes' cyclization. This is probably due to a slower cyclization rate of the free PTC glutamic acid, as it is not observed during cyclization of PTC glutamic acid in the protein. The mean of the yields at 40 and 60 minutes has been used in the calculation.

Table 3. Yield of different amino acids added to fibrinogen.

Amino acid	Added (μ moles)	Recovered (μ moles)	Yield (%)	Cyclization time (min.)	Solvent
Glycine	1.5	1.130	*75.5*	0	F
	1.5	1.116	74.4	20	
	1.5	1.097	73.1	40	
	1.5	1.088	72.5	60	
Tyrosine	1.5	1.148	*76.5*	0	F
	1.5	1.028	68.5	20	
	1.5	0.950	63.3	40	
	1.5	0.798	53.4	60	
Alanine	1.5	0.803	*53.5*	0	D, E
	1.5	0.781	52.1	30	
	1.5	0.775	51.7	50	
	1.5	0.759	50.6	70	
Threonine + Δ-threonine	1.5	0.764	50.9	0	H
	1.5	0.743	49.5	40	
	1.5	0.743	49.5	60	
	1.5	0.807	53.8	80	
Glutamic acid	1.5	0.742	49.5	20	F
	1.5	0.886	59.1	40	
	1.5	0.915	61.0	60	

However, it must be pointed out that the yield of glutamic acid might be influenced by the esterification, which seems to take place, to a small extent, between the γ-carboxyl group of PTH glutamic acid and some reactive residue in the protein.

The yield of the different amino acids is summarized in Table 3.

Results

A. *N-terminal amino acids in bovine fibrinogen*

The N-terminal amino acids were determined in 10 different preparations of purified fibrinogen (fraction I-4). PTH glutamic acid and PTH tyrosine were identified in several solvent systems (E, D, F, H). No other spots of ethyl acetate-soluble PTH amino acids could be detected if the PTC derivative of fibrinogen had been thoroughly washed with acetone. When the acetone washings had been omitted, traces of free glycine (about 0.04 *M* per 10^5 g of protein) were found even after dialysis of the fibrinogen for 24–30 hours. The amounts of N-terminal tyrosine and glutamic acid were the same, irrespective of whether or not the acetone washings had been omitted.

Arginine and histidine are not likely to be present as N-terminals in fibrinogen, as no spot with PTH absorption could be detected on chromatograms of the aqueous phase after cyclization (solvent E).

Table 4. N-terminal amino acids in fibrinogen.

Exp. no.	Moles amino acid per 10^5 g protein: Tyrosine solv. E	Tyrosine solv. F	Glu. acid solv. F	Glu. acid ethyl ester solv. G	Total protein (mg)	Moles amino acids per 10^5 g protein[a]	Cyclization time (min.)
1	...	...	...	...	65.0	3.40	60
2	...	...	...	...	136.0	1.86	60
3	...	...	...	...	99.7	1.51	60
4	0.423	...	0.320	0.065	258.0	...	60
5	0.437	...	0.321	0.028	326.0	1.93	60
6	0.441	...	0.385	0	197.3	...	60
7	0.411	0.436	0.270	...	157.8	...	10
	0.432	0.464	0.258	...	157.8	...	20
	0.449	0.431	0.254	...	157.8	...	40
	0.433	0.405	0.282	...	157.8	...	60
8	0.397	0.453	0.300	...	302.7	...	20
	0.401	0.447	0.287	...	90.9	...	40
	0.397	0.556	0.275	...	151.4	...	60
9	0.449	0.407	...	...	145.3	...	60
	0.425	0.447	0.323	...	145.3	...	30–60
10	0.461	...	...	...	186.7	...	60
11	0.435	0.473	0.382	...	121.1	...	60
12	0.470	0.419	0.266	...	134.1	...	60
13	0.408	...	0.244	...	141.2	...	60
14	0.442	...	...	...	141.2	...	60
15	0.431	0.434	0.266	...	154.8	...	60
Mean	0.430	0.448	0.296				
	0.437[b]		0.296[b]				

[a] Before chromatography.
[b] Uncorrected value.

As to the performic acid oxidized fibrinogen, no cysteic acid could be detected, whereas tyrosine and glutamic acid were clearly demonstrable, the yield of tyrosine being about 50% of the expected.

In Table 4 are listed the mean figures for the different experiments. The mean value in moles per 10^5 g of protein was found to be 0.437 for tyrosine and 0.296 for glutamic acid.

The molecular weight of human and bovine fibrinogen has been estimated to be about 340,000 by ultracentrifugation and light-scattering studies (Katz, Gutfreund, Shulman & Ferry, 1952; Caspary & Kekwick, 1954, 1957; cf. Edsall, 1954). Using 340,000 as the molecular weight and taking the yield of the terminal residues in the determination procedure into account, 1.94 moles of tyrosine and 1.68 moles of glutamic acid were calculated per mole of fibrinogen.

The lower value for glutamic acid can partly be explained by binding of the PTH derivative to the rest of the protein during cyclization, e.g. esterification with the hydroxyl groups of serine and threonine. In view of the ease with which PTH glutamic acid is esterified by ethanol, it was thought that treatment of the PTC protein

Table 5. N-terminal amino acids in bovine fibrinogen and fibrin.
(Fg = fibrinogen; F = fibrin.)

	Lorand & Middlebrook (1952) DNP method			Bailey & Bettelheim (1955) DNP method			Present authors (1957) PTC method		
Protein	Tyr.	Glu. acid	Gly.	Tyr.	Glu. acid	Gly.	Tyr.	Glu. acid	Gly.
Fg	1.5	0.7	—	2.3	1.3	—	1.9	1.7	—
	(2)	(1)		(2)	(1)		(2)	(2)	
F	1.4–1.6	—	3.0–3.7	2.3	—	2.7	1.9	—	3.9
	(2)		(4)	(2)		(2–3)	(2)		(4)

in the presence of ethanol would result in formation of the PTH glutamic acid ethyl ester. In some experiments, therefore, the water-phase left after extraction of the PTH amino acids was evaporated *in vacuo*, and the residue refluxed with 1 *N* HCl in 90% ethanol for several hours. After cautious neutralization, the solvent was evaporated *in vacuo*, and the phenylthiohydantoin extracted from the residue with ethyl acetate. On paper chromatograms (solvents E, D, F and G) a spot with the same R_f value as PTH glutamic acid ethyl ester was observed. Further evidence supporting the view that glutamic acid can be bound to the protein moiety is the observation that the yield of the phenylthiohydantoin increases with decreasing protein concentrations (Blombäck & Yamashina, 1957).

In the two experiments in Table 4, the sum of free acid and ester makes up between 80 and 90% of the tyrosine value. However, in one experiment (experiment 6, Table 4) no ester was obtained, but in this case the yield of the free acid was fairly high, amounting to 80% of the tyrosine value. Consequently, when the yields of the amino acids are taken into account, there seem to be equimolar amounts of N-terminal tyrosine and glutamic acid present in fibrinogen.

On the basis of these findings, it could be concluded that two tyrosyl and two glutamyl residues were present as the N-terminal amino acids in the fibrinogen molecule. A comparison of our figures with those of Lorand & Middlebrook (1952) and Bailey & Bettelheim (1955) obtained with the DNP method is shown in Table 5. The figures of the earlier workers were recalculated to a 340,000 unit weight of protein. The bracketed figures give the most probable numbers of N-terminal residues per mole.

The drastic conditions used for cyclization of the PTC amino acids result in splitting off of all amide nitrogen, if any is initially present in the N-terminal glutamyl residues. Actually, it was possible to show qualitatively that small amounts of glutaminyl residues were present when cleavage of the PTC protein was performed at pH 1–2 for 48 hours at room temperature, as described by Edman & Lauber (1956) for preparation of PTH glutamine from glutamine. It is remarkable, however, that the PTC tyrosyl residues in fibrinogen did not form PTH tyrosine under these mild conditions. By adjusting the system to standard conditions, the PTH tyrosine could be recovered. As a quantitative evaluation, a determination was carried out on the supernatant, after coagulation of the fibrinogen by thrombin since, as previously mentioned, the yield of PTH glutamic acid from glutamyl proteins was lower than that from glutamyl peptides.[1] Cyclization was first performed at pH 1–2 for

[1] Small amounts of non-coagulated protein were present in this system.

Table 6. N-terminal glutamine in bovine fibrinogen.

The amount of fibrinogen in the experiment was 218 mg.

Amino acid	μ moles of amino acid obtained: pH 1–2 48 hrs room temperature	μ moles of amino acid obtained: 1 *N* HCl 1 hr 100°C	Moles per 10^5 g protein	Per cent of total amino acid
Glutamine	0.080	—	0.037	27.6
Glu. acid	0.064	0.148	0.097	72.4
Total	0.144	0.148	0.134	100.0

48 hours at room temperature. After extraction of the PTH glutamine formed, the rest was again submitted to cyclization at 100°C in 1 *N* HCl for one hour. It can be seen from Table 6 that about 30% of the total glutaminyl–glutamyl residues obtained in this experiment could be identified as amide. Considerable destruction had, however, taken place, since the total yield was only 45% of the glutamic acid obtained from the corresponding amount of fibrinogen.

B. *N-terminal amino acids in bovine fibrin*

In bovine fibrin, glycyl residues replaced the glutamyl residues of fibrinogen. The figures obtained with different fibrin preparations are listed in Table 7. The amount of tyrosine was the same in fibrin as in a corresponding amount of fibrinogen. The amount of glycine was, however, twice that of tyrosine, thus indicating that, in fibrin, four glycyl residues have replaced the two glutamyl residues present in fibrinogen. The results with respect to the number of residues in fibrin reported by the different workers are shown in Table 5.

Since the fibrin clot was dissolved in strong urea solution prior to coupling with phenylisothiocyanate, the N-terminal amino acids in fibrinogen dissolved in this medium were also determined. However, urea was found not to affect the N-terminal amino acid pattern in fibrinogen.

Table 7. N-terminal amino acids in bovine fibrin.

Exp. no.	Moles amino acid per 10^5 g protein: Tyrosine solv. E	Moles amino acid per 10^5 g protein: Glycine solv. E	Total protein (mg)	Moles amino acids per 10^5 g protein[a]	Cyclization time (min.)	Other conditions
1	0.450	0.788	82.5	2.61	60	Thrombin: about 130 NIH units per mg
2	0.407	0.877	199.9	2.14	60	
3	0.394	0.863	152.4		60	
4	0.412	0.856	151.6		60	
5	0.490	0.892	107.8		60	Thrombin: 1380 NIH units per mg
Mean	0.431	0.855				

[a] Before chromatography.

In most experiments, the fibrinogen was coagulated by thrombin containing about 130 NIH units per mg. Furthermore, the result was confirmed on fibrin prepared by means of a purer thrombin preparation, which was an autoactivation product of prothrombin, containing 1380 NIH units of thrombin per mg (see Table 7).

C. *N-terminal amino acids in human, pig, sheep, goat, horse and dog fibrinogen*

The figures for the N-terminal amino acids in the different species are listed in Table 8. A feature common to the different species was the presence of tyrosyl residues, which amounted to 0.4–0.6 mole per 10^5 g of protein.

In human fibrinogen, approximately equal numbers of alanyl and tyrosyl residues were found, whereas in pig fibrinogen the relation between tyrosine and alanine was 1:2.

Sheep and goat fibrinogen differ from all species hitherto studied in that they have glycine as an N-terminal amino acid in addition to tyrosine and alanine. The same result was obtained both with fibrinogen prepared from citrated blood collected at the slaughter house, and with blood withdrawn by a venous cannula, the latter technique being employed to minimize the risk of partial conversion of the fibrinogen during the collection of the blood. The most probable relation between the amino acids was two tyrosyl, two alanyl and two glycyl residues in a 350,000 unit weight.

In horse and dog fibrinogen, threonine besides tyrosine could be identified as an N-terminal. Threonine was identified mainly as PTH dehydrothreonine. After correcting for the yield of PTH dehydrothreonine, tyrosine and threonine were calculated to be present in about equimolar amounts.

By means of light-scattering, the molecular weight of bovine, sheep and goat fibrinogen was estimated to be approximately 350,000. The value for horse fibrinogen was considerably higher, i.e., approximately 500,000 (Blombäck & Laurent, 1958).

In a 350,000 unit weight of fibrinogen from the different species, except the horse, there are most probably two tyrosyl residues, and the same number of tyrosyl residues are present in a 500,000 unit weight of horse fibrinogen.

D. *N-terminal amino acids in human, pig, sheep, goat, horse and dog fibrin*

The fibrinogen from all species was transformed into fibrin by means of bovine thrombin. The amount of tyrosine was practically the same in fibrin as in the corresponding amount of fibrinogen. In all species glycine appeared as an N-terminal residue and the alanyl or threonyl residues disappeared. The ratio of tyrosyl to glycyl residues was 1:2 in human, pig, sheep and goat fibrin. In dog fibrin the amount of glycine was somewhat lower; thus, the ratio of tyrosine to glycine was found to be 1.0:1.6. As distinguished from the other species, equimolar amounts of tyrosine and glycine were found in horse fibrin. The results are recorded in Table 8.

The glycyl residues in sheep and goat fibrin are not identical with those already present in fibrinogen. This is shown by an analysis of the N-terminal amino acids on the fibrin, as well as on the supernatant after complete coagulation by thrombin (Table 9). Since the most probable number of residues in fibrin are two tyrosyl and four glycyl, whereas six glycyl residues were present in the whole coagulation mixture, two glycyl and two alanyl residues are likely to have been split off by thrombin.

Table 8. N-terminal amino acids in fibrinogen and fibrin from different species.

Bracketed figures denote the relative amount of different amino acids referred to tyrosine.

	Moles amino acid per 10^5 g protein									
	Tyrosine		Alanine		Glycine		Glutamic acid + ethyl ester[b]		Threonine	
Proteins	Direct	Corr.[a]	Direct	Corr.[a]	Direct	Corr.[a]	Direct	Corr.[a]	Direct	Corr.[a]
Ox										
Fibrinogen	0.437	0.571 (1.00)					0.343	0.572 (1.00)		
Fibrin	0.431	0.563 (1.00)			0.855	1.132 (2.01)				
Man										
Fibrinogen	0.376	0.492 (1.00)	0.209	0.391 (0.80)						
Fibrin	0.382	0.499 (1.00)			0.706	0.935 (1.87)				
Pig										
Fibrinogen	0.351	0.459 (1.00)	0.472	0.882 (1.92)						
Fibrin	0.348	0.455 (1.00)			0.651	0.862 (1.89)				
Sheep										
Fibrinogen	0.376	0.492 (1.00)	0.229	0.428 (0.87)	0.306	0.405 (0.82)				
Fibrin	0.357	0.467 (1.00)			0.672	0.890 (1.91)				
Goat										
Fibrinogen	0.386	0.505 (1.00)	0.245	0.458 (0.91)	0.298	0.395 (0.78)				
Fibrin	0.386	0.505 (1.00)			0.716	0.948 (1.88)				
Horse										
Fibrinogen	0.304	0.398 (1.00)							0.176	0.346 (0.87)
Fibrin	0.283	0.370 (1.00)			0.287	0.380 (1.03)				
Dog										
Fibrinogen	0.374	0.489 (1.00)							0.237	0.466 (0.95)
Fibrin	0.388	0.507 (1.00)			0.593	0.785 (1.55)				

[a] Corrected with regard to the yields of the different amino acids as shown in Table 3.
[b] The figure for the ethyl ester is the mean of experiments 4 and 5, Table 4.

Table 9. Release of glycyl peptides during conversion of goat and sheep fibrinogen to fibrin.

	Relative amount of different N-terminal amino acids referred to tyrosine		
	Tyrosine	Alanine	Glycine
Sheep fibrinogen	1 (2)[a]	0.87 (2)	0.82 (2)
Fibrin plus supernatant after full conversion	1 (2)	0.78 (2)	2.63 (6)
Sheep fibrin	1 (2)	—	1.91 (4)
Goat fibrinogen	1 (2)	0.91 (2)	0.78 (2)
Fibrin plus supernatant after full conversion	1 (2)	0.92 (2)	2.66 (6)
Goat fibrin	1 (2)	—	1.88 (4)

[a] Bracketed figures denote the most probable number in the molecule.

E. *N-terminal amino acids in fibrins formed by the action of Reptilase and papain on bovine fibrinogen*

Qualitatively, no difference was found between these fibrins and those formed by the action of thrombin. The amount of the tyrosyl residues per unit weight of protein was the same as in normal fibrin, but that of glycyl residues was only half. Thus these fibrins resemble horse fibrin. The results are listed in Table 10. The results obtained after five and fifteen hours' incubation with Reptilase were the same.

Table 10. Reptilase and papain fibrin.

Bracketed figures denote the relative amount of different amino acids referred to tyrosine.

Enzyme	Per cent of total protein recovered in clot	Moles amino acid per 10^5 g protein					Cyclization time (min.)
		Tyrosine		Glycine		Other	
		Direct	Corr.[a]	Direct	Corr.[a]		
Reptilase	89.2	0.421	0.550 (1.00)	0.412	0.546 (0.99)		60
Papain	45.1	0.400	0.523 (1.00)	0.510	0.675 (1.29)	0.08	60

[a] Corrected with regard to the yields of the different amino acids as shown in Table 3.

Discussion

The observations on N-terminal amino acids in fibrinogen and fibrin made by Bailey & Bettelheim and by Lorand & Middlebrook, applying Sanger's method, have been confirmed by determinations using Edman's method on purer fibrinogen preparations. Differences in quantity are, however, present.

The varying results obtained by Sanger's method are evidently due to destruction of DNP amino acids during hydrolysis of DNP proteins, the extent differing for each amino acid and each protein. The destruction numbers obtained by determining the yield of free DNP amino acids after hydrolysis in 6 *N* HCl, with or without the presence of protein, are not always applicable to the N-terminal residues in an unknown protein, as has been pointed out by many workers. As far as fibrinogen is concerned, the difference in the number of terminal residues obtained by different authors with the DNP method can principally be ascribed to the different destruction numbers used (Bailey & Bettelheim, 1955). In this respect, Edman's method has proved to be more reliable. The estimation of the over-all yield was carried out on free amino acids in the presence of a certain amount of protein. This was because, in the absence of protein, some of the PTC and PTH amino acids are appreciably destroyed by the side-reaction products formed during the determination procedure, under the conditions used by us. The yields thus obtained could be used for correction of the experimental values for the different N-terminal amino acids. The loss of PTH amino acids could be ascribed mainly to the loss in paper chromatography, indicating that destruction of PTH amino acids occurring during the whole determination procedure is small. It has, however, been shown that the PTH derivative of at least one dicarboxylic amino acid can to a small extent be bound to reactive groups in the protein moiety.

As seen in Table 5, our values for the tyrosyl residues agree with those obtained by Lorand & Middlebrook (1952) and by Bailey & Bettelheim (1955). There is, however, a difference as to the number of glycyl and glutamyl residues. Furthermore, it could be shown that, in bovine fibrinogen, at least part of the γ-carboxylic groups in glutamic acid were in the amide form.

Our investigations on the N-terminal amino acids have also been extended to fibrinogen and fibrin from different species. With the methods used for purification of fibrinogen, it was possible to obtain fibrinogen preparations of high purity of about the same degree from the different species. No spots which could be ascribed to protein impurities were seen on paper chromatograms.

Lorand & Middlebrook (1953) found, in human fibrinogen, alanyl and tyrosyl residues as N-terminals (one tyrosyl in 2.2×10^5 g and one alanyl in 3×10^5 g), the alanyl residue being replaced by two or three glycyl residues in the corresponding amount of fibrin. Bailey & Rosemeyer (cited by Sanger, 1955) reported tyrosyl and threonyl residues in horse fibrinogen, but no quantitative evaluation was given. These workers used Sanger's technique.

It is evident from the present results that the tyrosyl residue, whatever its function in the polymerization process may be, is a feature common to fibrinogen and fibrin from all species investigated. It is also apparent that the amount of tyrosyl residues in fibrinogen and in fibrin is about the same in all species, except in the horse. In addition to tyrosine, another N-terminal amino acid is always present in fibrinogen. In bovine fibrinogen, it consists of glutamic acid, in horse and dog of threonine, and in human, pig, sheep and goat of alanine. In sheep and goat fibrinogen, glycine is present as a third N-terminal amino acid. Since the pattern of the terminal amino acids varies, the additional ones do not seem to be included in the specific requirements for the transformation of fibrinogen to fibrin by the thrombic enzyme. It was remarkable to find glycine as the third N-terminal amino acid in sheep and goat fibrinogen. As the amount was so constant in the two species and in different preparations, provided that dialysis was thoroughly performed before coupling, the

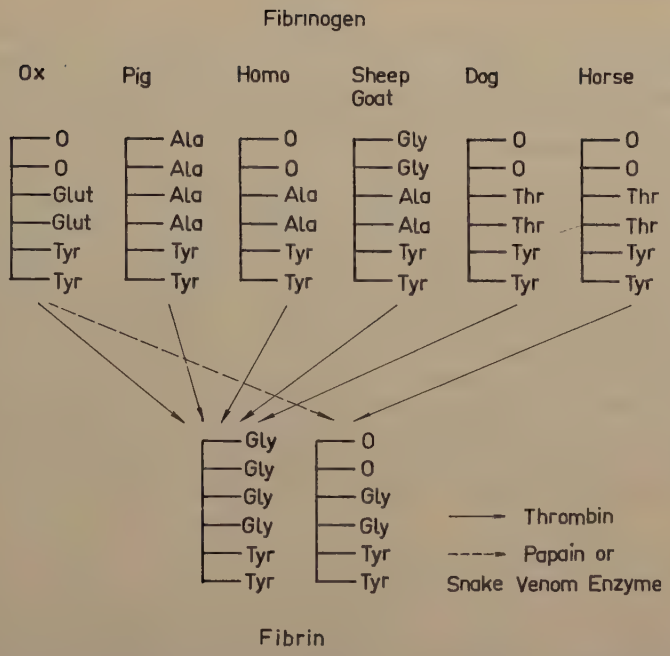

Fig. 2. N-terminal amino acids in fibrinogen and fibrin from different species.

glycine cannot be ascribed to contamination during the preparation procedure. In addition, the PTC protein was washed with acetone, this process being known to remove free PTC amino acids.

In the scheme shown in Fig. 2, a general pattern has been suggested for the conversion of fibrinogen to fibrin. The circles indicate chains with no free N-terminal groups, e.g., cyclic chains, because no other amino acids have hitherto been found. The number of chains is the most probable in a 350,000 unit weight of human, bovine, pig, sheep, goat and dog fibrinogen, and in a 500,000 unit weight of horse fibrinogen.

As suggested in the figure, different kinds of peptides seem to be split off from the fibrinogen of the various species. In bovine fibrinogen, two glutamyl and two cyclic peptides are released by the action of thrombin, in pig four alanyl peptides, in human two alanyl and two cyclic peptides, in sheep and goat two glycyl and two alanyl peptides, in dog two threonyl and two cyclic peptides, and in horse two threonyl peptides.

Among the proteolytic enzymes, thrombin seems to have an extremely narrow specificity of action, as has been stated by Bailey & Bettelheim (1955). In a molecule containing some thousand bonds, only four are split during the conversion of fibrinogen to fibrin, as judged by the actual N-terminal analysis. The peptide bonds in ovalbumin or rabbit myosin are not susceptible to thrombin, nor is the glutamyl–arginyl–glycyl–phenylalanine sequence of the B chain of insulin (Bailey & Bettelheim, 1955).

Bovine fibrinogen, at any rate, can also be converted to fibrin by other proteolytic enzymes, e.g., papain and snake-venom enzymes (Eagle & Harris, 1937; Eagle, 1937).

Papain is known to have a relatively broad specificity of action, and will readily digest fibrinogen and fibrin into a large number of fragments. Consequently, the papain fibrin initially formed under the conditions mentioned above will gradually be proteolyzed into soluble peptides. However, the insoluble product of proteolysis, the papain fibrin, qualitatively shows the same N-terminal residues as thrombin fibrin, provided that the fibrin has been thoroughly washed to remove secondary split products. Reptilase, the coagulating enzyme in the venom from *Bothrops jararaca*, has a coagulating and proteolytic activity like that of thrombin towards fibrinogen and synthetic substrates (Blombäck, Blombäck & Nilsson, 1957). This also applies to the N-terminal amino acids. More limited proteolysis seems, however, to occur in the transformation to fibrin by Reptilase or papain than by thrombin, since only two glycyl residues appear. Thus, if proteolysis is necessary for the subsequent polymerization, splitting of only a few of the available peptide bonds seems to be indispensable for initiating polymerization under certain conditions, in view of the fact that only two of the four bonds in bovine fibrinogen susceptible to thrombin are split by Reptilase or papain. It is noteworthy that horse fibrin formed by thrombin is of the same type.

SUMMARY

Quantitative N-terminal amino acid analysis of fibrinogen and fibrin from seven different species has been made by means of the phenylthiocarbamyl method of Edman. The analytic procedure suitable for these proteins is described in detail. In all species, both the fibrinogen and the fibrin contained 0.4 to 0.6 moles of N-terminal tyrosine per 10^5 g of protein. In addition to tyrosine, another N-terminal amino acid was always present in fibrinogen. In bovine fibrinogen, it consisted of glutamic acid and/or glutamine, in horse and dog fibrinogen of threonine, and in human, pig, sheep and goat fibrinogen of alanine. Furthermore, in sheep and goat fibrinogen, glycine was present as a third N-terminal amino acid. In fibrin, these additional amino acid residues were replaced by glycyl residues, indicating a splitting off of different peptides in the different species.

The fibrin formed from bovine fibrinogen by the action of a snake-venom enzyme (Reptilase) or papain had the same N-terminal amino acids as normal fibrin. However, the ratio of tyrosine to glycine in this fibrin was 1:1 instead of 1:2 in normal fibrin.

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By Birger Blombäck

With 11 figures in the text

The enzymatic nature of the action of thrombin was recognized early (Schmidt, 1872; Hammarsten, 1876). The hypothesis that a limited proteolysis might be involved in the reaction has been put forward by several authors during the past 70 years (cf. Morawitz, 1905; Bailey & Bettelheim, 1955; Scheraga & Laskowski, 1957). The coagulation of blood was suggested by Waldschmidt-Leitz, Stadler & Steigerwaldt (1928) to involve proteolysis, in which process thrombin was believed to be a "trypsin-kinase"-like enzyme. Fuchs & Zakrzewski (1934) reported that during the conversion of fibrinogen to fibrin there was an increase in non-protein nitrogen in the coagulation mixture, an increase which, in fact, was ascertainable already before visible coagulation. Presnell (1938), by means of formol titration, found an increase in the number of free amino groups during the coagulation of fibrinogen.

It was clearly shown by Eagle (1937) and Eagle & Harris (1937) that different proteolytic enzymes had blood-coagulating activity. Thus, trypsin could transform prothrombin to thrombin, whereas papain induced a fibrinogen-fibrin transformation. Of several snake venom enzymes investigated, some were shown to affect the fibrinogen-fibrin transformation and others the prothrombin-thrombin conversion. On the basis of these observations, the authors suggested proteolysis to be of importance in normal blood coagulation as well. In 1945, Laki & Mommaerts stated that a "two-step" eaction was involved in the transformation of fibrinogen to fibrin in the presence of thrombin. They found that, although no coagulation occurred at pH values about 5, the fibrinogen was changed in some way by the action of thrombin, since the coagulation time after neutralization was shorter for a fibrinogen solution that had been preincubated with thrombin. Laki (1951) suggested that thrombin prepares the fibrinogen molecules in some way for the polymerization which occurs only when the ionic milieu is suitable. These changes in the fibrinogen at low pH values were also effected by thrombic enzymes from snake venoms (Janszky, 1949).

However, more definite evidence of a limited proteolytic activity of thrombin was not given until 1951, when Bailey, Bettelheim, Lorand & Middlebrook, by means of Sanger's dinitrophenyl method, showed a difference between the N-terminal amino acids of bovine fibrinogen and fibrin. Thus, the glutamyl residues in bovine fibrinogen were replaced in fibrin by glycyl residues. This indicated a splitting off of peptide material during the transformation. Furthermore, the earlier indications that non-protein nitrogen was released during and before the coagulation of fibrinogen were confirmed (Lorand, 1952; Kowarzyk, 1952; Laskowski, Donelly, van Tijn & Scheraga, 1956).

In conformity with the indications given by the studies of Laki & Mommaerts, evidence was obtained from N-terminal analysis that thrombin could act, although slowly, at low pH values, where no coagulation occurred (Bailey and Bettelheim, 1955). Thorough analyses of the N-terminal amino acids in bovine fibrinogen and fibrin have been made since 1951, using Sanger's technique (Bettelheim & Bailey, 1952; Lorand & Middlebrook, 1952; Bailey & Bettelheim, 1955).

Lorand & Middlebrook (1953) found that the N-terminal amino acid pattern of human fibrinogen differed from that of bovine fibrinogen. The N-terminal amino acids in fibrin were, however, the same in both species.

Applying Edman's phenylthiocarbamyl method, Blombäck & Yamashina (1957, 1958) analyzed the N-terminal amino acids in fibrinogen and fibrin from several animal species. These studies suggested that different kinds of peptides are split off from the fibrinogen of the various species by the action of thrombin.

From the clot supernatant of bovine fibrinogen, Lorand (1952) obtained an acidic peptide having glutamic acid as the N-terminal group. The isoelectric point was between pH 3 and 4 (Lorand, 1954). However, Bettelheim & Bailey (1952) and Bettelheim (1956) isolated by means of electrophoresis two peptides, tentatively named A and B. The A peptide, containing the N-terminal glutamic acid of fibrinogen, had an isoelectric point of about pH 3.1–3.2, thus resembling the peptide obtained by Lorand. No N-terminal amino acid was found in peptide B. The A and B peptides could also be fractionated by partition chromatography (Bettelheim, 1956). Peptide B, in contrast to peptide A, was shown by Bettelheim (1954) to contain tyrosine. The phenolic group of tyrosine was, however, not free but esterified by sulphuric acid. Peptide A seemed to be released by the action of thrombin at a higher initial rate than peptide B.

By chromatography on 2 % cross-linked Dowex-50, Blombäck & Vestermark (1958) also isolated two peptides from the clot supernatant of bovine fibrinogen. One peptide contained N-terminal glutamic acid, whereas no N-terminal amino acid residue was found in the other. The latter peptide, however, contained tyrosine-O-sulphate. The two peptides were also shown to be released at different initial rates during coagulation. The release of peptide A could be correlated to fibrin formation. These peptides seem to be identical to those isolated by Bettelheim and Bailey.

In the studies reported here, the release of peptide material from bovine fibrinogen by the action of bovine thrombin under different experimental conditions has been further investigated, by means of N-terminal determinations. Thus, a study has been made of the dependence of this process on pH, temperature, ionic strength, enzyme and substrate concentration, as well as its inhibition by heparin and its plasma co-factor. In addition, the action of a snake-venom enzyme (Reptilase) has been investigated.

Material and methods

Bovine fibrinogen.—Fibrinogen was prepared according to Blombäck & Blombäck (1956). The coagulability of the preparations used was between 95 and 99 %. Both protein concentration and coagulability were determined spectrophotometrically (Blombäck, 1958).

Thrombin.—The preparation of the thrombin used has been described by Blombäck & Yamashina (1958). The activity was about 130 NIH units/mg.

Heparin.—Heparin Vitrum, Stockholm, was used. The activity of the preparation was 94 international units/mg.

Reptilase.—This enzyme was obtained from Pentapharm A.G., Basel, in ampoules of 1 ml. The preparation contains the coagulating enzyme of the venom of *Bothrops jararaca*. The activity corresponded to approximately 1.1 NIH thrombin units/ml.

Heparin co-factor.—Citrated ox plasma was used as a source of heparin co-factor. Nine parts of blood were mixed with one part of 3.8 per cent trisodium citrate ($\times 2$ H_2O).

N-terminal amino acid analysis.—Edman's phenylthiocarbamyl method was applied. The analytic technique has been described in detail by Blombäck & Yamashina (1958). After washing the phenylthiocarbamyl derivatives twice with benzene, the PTC proteins were precipitated by adding acetone to a concentration of 80 %. The precipitate was then thoroughly washed twice with aqueous acetone for quantitative removal of the PTC derivative of peptide A. The amounts of phenylthiohydantoins have been expressed in μmoles. As the amount of N-terminal tyrosine is the same in fibrinogen and the corresponding amount of fibrin, the N-terminal glycine (μmoles) appearing during the thrombin action has usually been expressed in per cent of the N-terminal tyrosine (μmoles). No correction for the yield of these amino acids has been made in this investigation, since the yield of glycine and tyrosine is practically the same. The glutamic acid has usually been expressed in per cent of the initial amount in fibrinogen.

Experimental and results

1. *N-terminal amino acids during conversion of fibrinogen to fibrin by bovine thrombin*

In a preliminary experiment, the N-terminal amino acids were determined at different times in the coagulation process in a fibrinogen-thrombin system. The experiment shown in Fig. 1 was performed at pH 6.3–6.4, ionic strength 0.17. The protein concentration was 0.17 per cent and the total amount of protein in each determination was 150 mg. All experiments were performed at room temperature (20–22°C).

The experimental procedure was briefly as follows. Into several beakers of the same size was put 30 ml of a 0.5 per cent fibrinogen solution in 0.3 *M* sodium chloride. To the fibrinogen solution was then added 60 ml of a phosphate buffer, ionic strength 0.1. (The composition of this buffer was 0.005 *M* Na_2HPO_4, 0.010 *M* KH_2PO_4 and 0.075 *M* NaCl.) The transformation was started by adding under stirring a thrombin solution (10 NIH units/ml) to a concentration of 0.05 NIH units/ml. In this system the first fibrin threads appeared after 5–6 minutes. This time was denoted as the coagulation time of the system. The reaction was stopped at different times by coupling the fibrinogen by the addition of phenylisothiocyanate (4 ml) in pyridine (90 ml). In the zero-time determination, the coupling reagents were added prior to addition of thrombin. When a clot had formed in a beaker, it was quickly collected on a glass rod before the coupling reagents were added to the supernatant. The clot was immediately dissolved in a solution of 40 % urea in 0.15 *M* tris buffer, pH 9.0–9.5, to a protein concentration between 0.1 and 0.7 %. This solution was used for coupling. The coupling and further preparation of the phenylthiohydantoin derivatives of the amino acids have been described in detail previously (Blombäck & Yamashina, 1958).

During the action of thrombin on fibrinogen, glycine appeared as an N-terminal amino acid immediately after addition of thrombin to the fibrinogen solution, thus

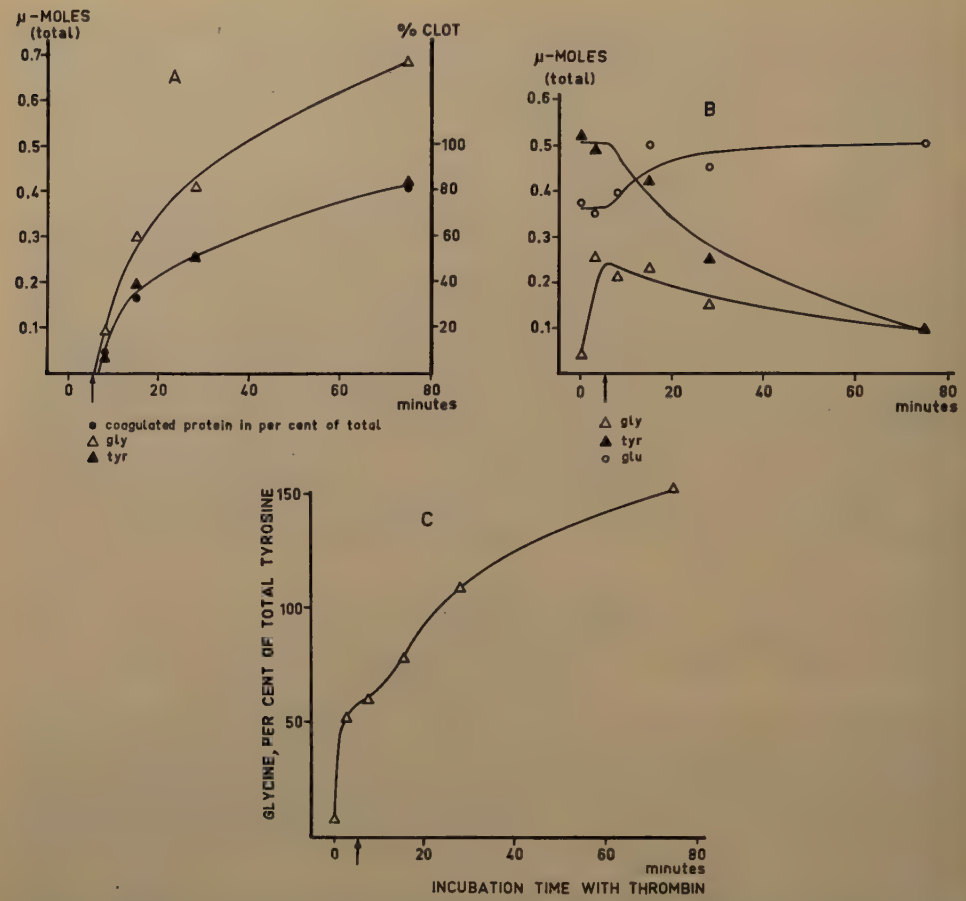

Fig. 1. N-terminal amino acids during conversion of fibrinogen to fibrin. *A*, N-terminal amino acids and protein content of clots collected at different times. *B*, N-terminal amino acids in the supernatant after removal of the clot. *C*, N-terminal glycine in clot plus supernatant, expressed in per cent of total tyrosine. △, gly; ▲, tyr; ○, glu; ● coagulated protein in per cent of total. Arrow indicates the coagulation time.

indicating formation of an intermediary product before visible coagulation. In this experiment, as much as 25 % of the total N-terminal glycine available appeared before any coagulation occurred. After fibrin formation had started, there was a slow decrease in the N-terminal glycine in the clot supernatant, and N-terminal glycine appeared in the fibrin. The decrease in N-terminal tyrosine in the solution ran parallel with the appearance of tyrosine end-groups in the fibrin formed. The sum of N-terminal tyrosine in fibrin and clot supernatant was thus nearly constant throughout the process.

As a consequence of the splitting off (before coagulation) of peptide A, which contains all the glutamyl residues of the original fibrinogen, the N-terminal glutamic acid was constant in the clot supernatant. The somewhat lower figure found for

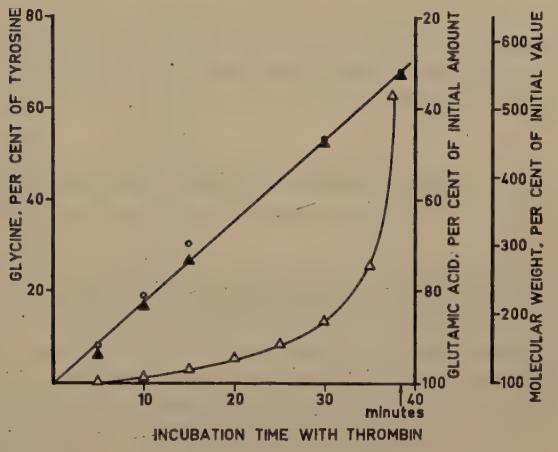
GLYCINE, PER CENT OF TYROSINE
GLUTAMIC ACID, PER CENT OF INITIAL AMOUNT
MOLECULAR WEIGHT, PER CENT OF INITIAL VALUE
INCUBATION TIME WITH THROMBIN
minutes

Fig. 2. Changes in protein N-terminal amino acids and light-scattering in the fibrinogen solution after addition of thrombin. ○, gly; ▲, glu; △, molecular weight in per cent of initial value. Arrow indicates the coagulation time.

glutamic acid in the early stage of transformation is probably due to a somewhat lower yield of the phenylthiohydantoin derivative of glutamic acid in the presence of high concentrations of protein, as was suggested in a preliminary note on this subject (Blombäck & Yamashina, 1957).

It can be inferred from Fig. 1 *C* that the appearance of the N-terminal glycine seems to take place in two steps. A satisfactory explanation of the biphasic curve is offered by the fact that peptides A and B are split off at different initial rates (Blombäck & Vestermark, 1958). Thus, peptide A is initially released at a far higher rate than peptide B, whereas later in the transformation process, the relative rate is in favour of peptide B. When, on the contrary, fibrinogen is converted to fibrin by means of Reptilase, which only splits off the glutamyl peptide (A) (see Part 4), the appearance of N-terminal glycine seems to take place in one step only.

The release of N-terminal glycine before visible coagulation is clearly demonstrated in Fig. 2. Transformation was performed in citrate buffer, pH 6.3–6.4, ionic strength 0.3 (0.3 *M* sodium chloride and 0.003 *M* citrate). The fibrinogen concentration was 0.12 %. The thrombin concentration in this experiment was 0.04 NIH units/ ml. The reaction was also studied by measurement of the light-scattering,[1] as described by Blombäck & Laurent (1958). In other respects, the experimental procedure was the same as that described above.

In this experiment, about 35 % of the total glycine appeared before any coagulation occurred. It is also seen that, concomitant with the release of glycyl residues, a decrease in protein N-terminal glutamic acid took place. The appearance of N-terminal glycine, expressed in per cent of the N-terminal tyrosine, closely followed the release of glutamyl residues expressed in per cent of the glutamyl residues originally present (Fig. 2). This indicates that only peptide A, which contains N-terminal glutamyl residues, is released during this early stage. During this release of N-terminal glycine, a lag period preceded the sudden increase in polymerization rate, which led to coagulation. Obviously, at least at the low pH value in question, considerable formation of

[1] The measurements were performed at this institute by Dr. T. Laurent.

an intermediary product had taken place before any appreciable polymerization had occurred. At higher pH values, however, the increase in molecular weight, as measured by light-scattering, runs parallel with the appearance of glycyl residues (Blombäck & Laurent, 1958).

2. *Proteolytic activity of bovine thrombin in relation to pH, ionic strength, temperature, and enzyme and substrate concentrations*

In this series of experiments, thrombic digestion was usually performed in tris-imidazol buffers, containing 0.017 *M* of each tris, and imidazol and further sodium chloride to the desired ionic strength. When not otherwise stated, the ionic strength of the digestion mixture was 0.2, and the protein concentration 0.17 %. The thrombin concentration was between 0.006 and 0.04 NIH units/ml. The measurements were usually made at room temperature (20–22°C). When not otherwise stated, digestion was performed at pH 9.0 only. At this pH, a clot is rarely formed and, if it is formed, it dissolves on coupling the sample with phenylisothiocyanate in pyridine. The experimental procedure was briefly as follows. Into several beakers of the same size was put 15 ml of a 0.5 % fibrinogen solution in 0.3 *M* sodium chloride. Thirty ml of tris-imidazol buffer of the desired pH and of ionic strength 0.15 was then added. The reaction was stopped at different intervals by coupling with phenylisothiocyanate in pyridine (Blombäck & Laurent, 1958).

pH optimum

As has previously been shown (Blombäck & Laurent, 1958), the pH optimum for the proteolytic activity of thrombin is between 8.5 and 9.0 in tris-imidazol buffer of ionic strength 0.2 (Fig. 3). The glutamyl residues were released at all pH values at

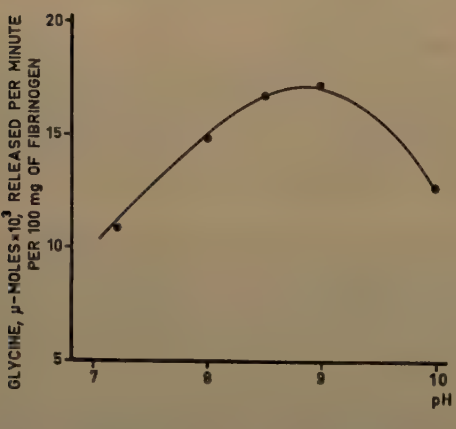

Fig. 3. Rate of appearance of N-terminal glycine at different pH values. Incubation time with thrombin: 20 minutes. Thrombin concentration 0.02 NIH units/ml.

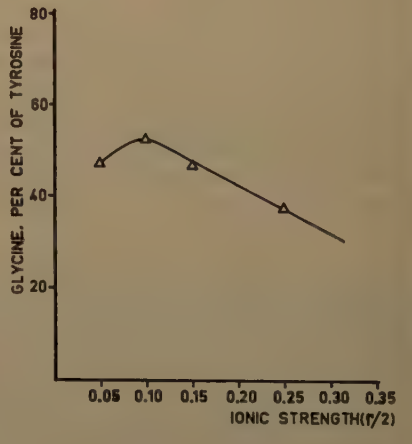

Fig. 4. Appearance of N-terminal glycine in the fibrinogen solution at different ionic strengths. Incubation time with thrombin: 20 minutes. Thrombin concentration: about 0.03 NIH units/ml.

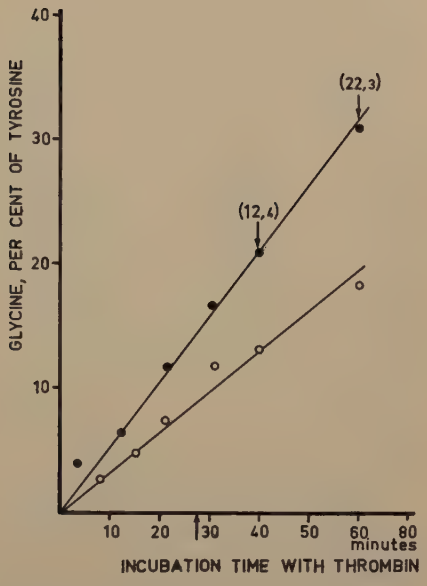

Fig. 5. Appearance of N-terminal glycine in the fibrinogen solution at different ionic strengths. ●, ionic strength 0.2; ○, ionic strength 0.3. The arrow on the abscissa indicates the coagulation time in the system of ionic strength 0.2. The bracketed figures denote the amount of fibrin formed in per cent of initial fibrinogen.

approximately the same rate as the glycyl residues appeared. The pH optimum is the same as for the esterase activity of thrombin measured on tosylarginine methyl ester in tris buffer (Sherry & Troll, 1954). This optimum does not, however, coincide with that for optimal visible coagulation, the latter being much lower, i.e., about pH 6.5 at the thrombin concentration in question.

Ionic strength

An increase in ionic strength (from 0.1 to 0.3) in a fibrinogen-thrombin system at about pH 7 is associated with a continuous increase in coagulation time.

Astrup (1944) demonstrated that the coagulation time of fibrinogen after addition of thrombin was markedly dependent on the ionic strength of the solution. The same observation was made by Edsall & Lever (1951).

The proteolytic activity of thrombin is also influenced by variations in electrolyte concentration (Fig. 4). Under the experimental conditions described above, the optimal ionic strength is about 0.1.

In the experiments recorded in Fig. 5, the release of N-terminal glycine was followed in coagulation mixtures of different ionic strengths. The transformation was performed in phosphate buffers of pH 6.3–6.4, ionic strength 0.2 and 0.3, respectively. In both series the protein concentration was 0.17 %, and thrombin was added to a concentration of 0.006 NIH units/ml. In the solution of ionic strength 0.2, coagulation occurred 28 minutes after the addition of thrombin, and in the last determination of this series (at 60 minutes), 22 % of the fibrinogen had been converted to fibrin. The clot formed was dissolved by addition of urea to the coupling mixture to a concentration of about 5 %. In the series with higher electrolyte concentration, no coagulation occurred. As can be seen, proteolysis is inhibited almost proportionately to the increase in ionic strength. It is further evident that the polymerization process

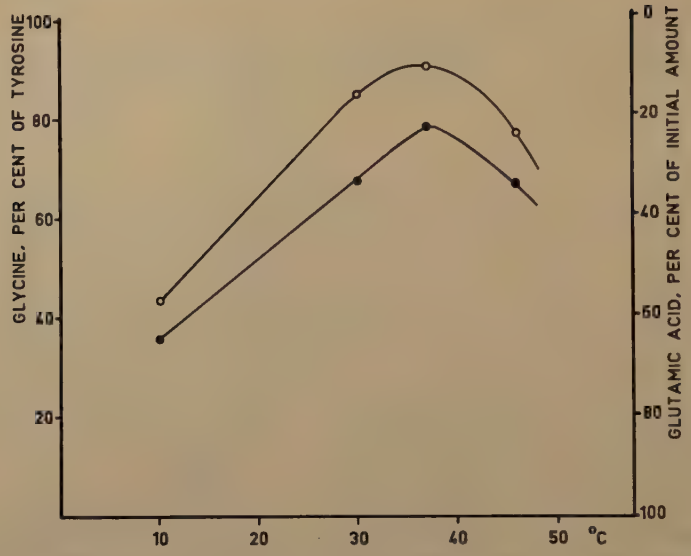

Fig. 6. Appearance of N-terminal glycine and disappearance of protein N-terminal glutamic acid in the fibrinogen solution at different temperatures. ○, gly; ●, glu. Incubation time: 20 minutes. Thrombin concentration: 0.02 NIH units/ml.

is influenced by the increase in ionic strength in a different way to proteolysis since, in the incoagulable system, proteolysis could proceed to a greater extent without coagulation than in the system of lower ionic strength.

Temperature

The proteolytic activity of thrombin is dependent in a characteristic manner on the temperature of the system (Fig. 6). The optimum activity is reached at +30 to +40°C. At temperatures above +40°C, there is a decrease in activity.

It is evident from Fig. 6 that the release of the glutamyl peptides runs parallel with the appearance of glycyl residues. Thus, it is mainly changes in the rate of release of peptide A that are measured.

The influence of temperature on the coagulation time of fibrinogen by thrombin was studied by Alexander Schmidt (1872). He found that the coagulating activity of thrombin increased with increasing temperature and reached a maximum at about 37°C. At higher temperatures there was an inhibition of coagulation.

Enzyme concentration

The experiments were performed at two enzyme concentrations, i.e., 0.02 and 0.04 NIH units/ml. It is evident that the initial rate of release of N-terminal glycine was proportional to the enzyme concentration (Fig. 7). The good correlation between the appearance of N-terminal glycine and the release of glutamyl residues in the early stage of conversion indicates that the initial rate is mainly an expression of the

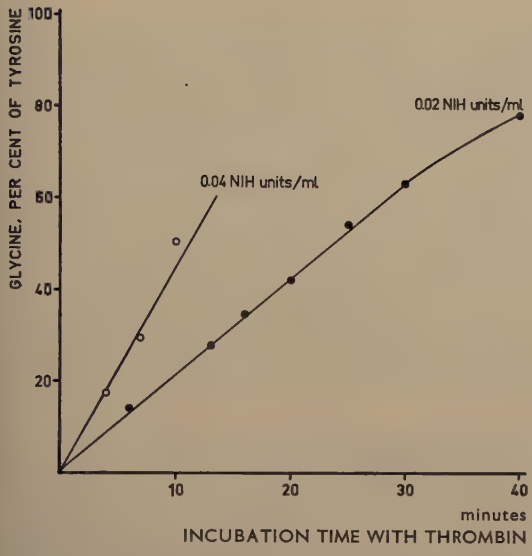

Fig. 7. Appearance of N-terminal glycine in the fibrinogen solution at different enzyme concentrations. •, 0.02 NIH units/ml; ○, 0.04 NIH units/ml.

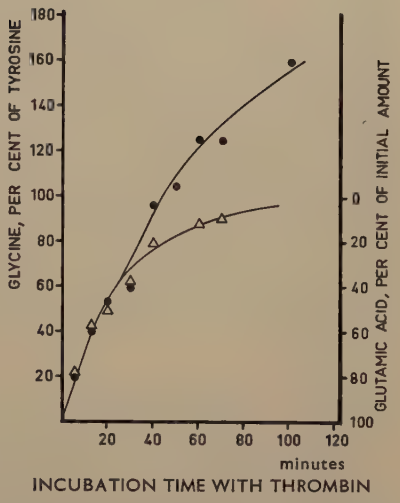

Fig. 8. Appearance of N-terminal glycine and release of N-terminal glutamic acid. Thrombin concentration: 0.02 NIH units/ml. △, glu;. •, gly.

release of peptide A (Fig. 8). When in this experiment the digestion was continued for longer than 30 minutes, by which time more than 30 % of the total glycyl residues in fibrin had appeared, the release of peptide A became successively slower. This was indicated by the levelling off of the release of glutamyl residues.

Under the present experimental conditions, 1 NIH unit (8 γ) of thrombin released approximately 0.1 μmoles of N-terminal glycine in 10 minutes in the initial stage of the reaction.

In contrast to proteolysis, the coagulation time in a fibrinogen–thrombin system is not a linear function of thrombin concentration (cf. Astrup, 1944).

Substrate concentration

As shown in Fig. 9, the proteolytic activity of thrombin, as measured by the appearance of N-terminal glycine, was dependent on the fibrinogen concentration. The appearance of glycyl residues was accompanied at all concentrations, except the lowest, by a release of approximately the same number of glutamyl residues, indicating that mainly peptide A had been split off at the higher concentrations.

The coagulation time of a fibrinogen–thrombin system is also dependent on the concentration of the substrate. Thus, the coagulation time after addition of a certain amount of thrombin to a fibrinogen solution at pH 7.0 decreases as the substrate concentration is increased from zero to about 0.3 %.

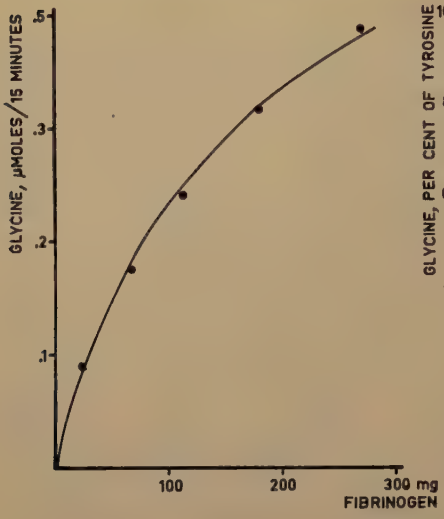

Fig. 9. Appearance of N-terminal glycine at different substrate concentrations. Incubation time with thrombin: 15 minutes. Thrombin concentration: 0.02 NIH units/ml. Volume: 45 ml in all samples.

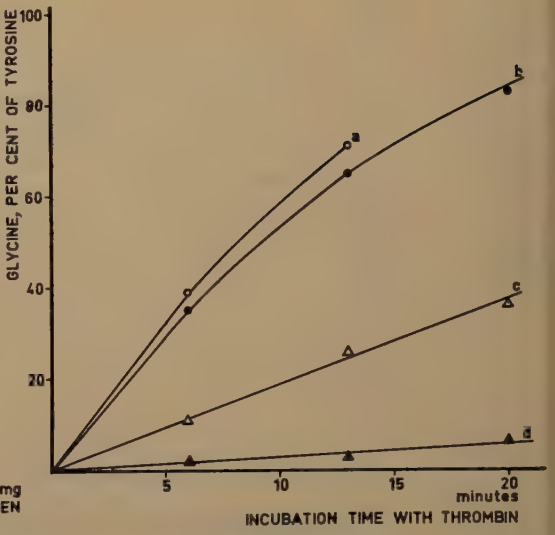

Fig. 10. Inhibition of proteolytic activity of thrombin by heparin and its plasma co-factor. *a*, Thrombin + plasma; *b*, thrombin; *c*, thrombin + heparin; *d*, thrombin + heparin + plasma.

3. *Inhibition of the proteolytic activity of bovine thrombin by heparin and its co-factor in plasma*

The inhibition studies were performed in tris-imidazol buffer, pH 7.4, ionic strength 0.15. The protein concentration was 0.2 % and the total amount of protein was 75 mg in each determination. Thrombin was added at zero time to a concentration of 0.04 NIH units/ml. All experiments were made at room temperature. Bovine plasma was used as the source of heparin co-factor, since the co-factor activity has been found to be high in bovine plasma (Blombäck, Blombäck & Wallén, 1955). The amount of plasma added was small, and the N-terminal amino acids derived from it did not interfere with the determination of N-terminal glycine. Four series of experiments were performed. In each series, determinations were made at three different digestion times.

The experimental procedure was as follows. Into several beakers of the same size were put 12.5 ml of 0.6 % fibrinogen solution in 0.15 *M* sodium chloride and 25 ml of tris-imidazol buffer, pH 7.4, ionic strength 0.15. To the beakers in the first series (*a*) was added 0.25 ml of ox plasma and 0.5 ml of water, to the second series (*b*) 0.75 ml of water, to the third (*c*) 0.5 ml of a heparin solution containing 390 international units/ml and 0.25 ml of water. The heparin concentration in the final mixture was then 5 units/ml. To the fourth series (*d*) was added 0.5 ml of heparin solution and 0.25 ml of ox plasma. The heparin and plasma were incubated in the fibrinogen solution for 10 minutes.

After the addition of thrombin, visible coagulation took place after 5 minutes in the first and second series (*a*, *b*), and after 11 minutes in the third (*c*). In the fourth series (*d*), the coagulation time exceeded 20 minutes. Digestion was interrupted at

different times by coupling the fibrinogen by phenylisothiocyanate. If a clot had formed, it was collected on a glass rod just before the phenylisothiocyanate was added to the clot supernatant. The clot was dissolved in a small volume of 40 % urea in tris buffer pH 9.0, and this solution was then combined with the clot supernatant.

The experiments are recorded in Fig. 10. Judging by the rate of appearance of N-terminal glycine, plasma alone, in the amounts in question, had no inhibitory action on thrombin (curve *a*). When heparin was added to the fibrinogen–thrombin system, it produced an appreciable inhibition of proteolysis, amounting to about 65 % (curve *c*). In the experiments where heparin together with its plasma co-factor was added to the fibrinogen-thrombin system, the proteolytic activity of thrombin was inhibited by more than 90 % (curve *d*).

It can be concluded from these experiments that heparin and especially heparin plus its co-factor are, under the conditions described, potent inhibitors of the proteolytic activity of thrombin, as well as of coagulation.

4. *Proteolytic action of Reptilase on bovine fibrinogen*

It has been found that Reptilase, the coagulating enzyme of the venom of *Bothrops jararaca*, exerts a specifically thrombin-like activity on blood coagulation (Blombäck, Blombäck & Nilsson, 1957; Hohnen, 1957). This can also be shown by N-terminal amino acid analysis. However, the molar ratio of tyrosine to glycine in Reptilase fibrin is 1:1, instead of 1:2 in the thrombin fibrin. This indicates that only peptide A is released by Reptilase (Blombäck & Yamashina, 1958).

By chromatography of the clot supernatants after coagulation of bovine fibrinogen by Reptilase it has, in fact, been shown that only peptide A is split off by this enzyme. In Fig. 11 is shown a chromatogram of a clot supernatant after 185 minutes' incubation of fibrinogen with Reptilase. The second small peak that appears after long incubation times may be peptide B. The rate of its release is, however, very slow. The amounts of peptide A released, as well as the amounts of fibrin formed at the different incubation times, are shown in Table 1. The yield of peptide A has been expressed as the quantity of human albumin giving the same colour with the combined biuret–phenol reagent of Lowry, Rosebrough, Farr & Randall (1951). The N-terminal analysis of the fibrin formed during different incubation times is also recorded in the table. As can be seen, the molar ratio of tyrosine to glycine in the fibrin formed is 1:1 throughout the experiment.

The A peptides split off by Reptilase or by thrombin were eluted from the columns with 0.1 *M* sodium acetate buffer, pH 4.4, at approximately the same effluent vo-

Table 1. Transformation of bovine fibrinogen by Reptilase.

Exp. no.	Incubation time with Reptilase (min.)	Fibrin formed (mg)	Peptide A released (mg)	N-terminal amino acids in fibrin formed, moles per 10^5 g protein	
				Glycine	Tyrosine
1	0	0	0	0	0
2	57	149	1.57	0.40 (0.91)	0.44 (1)
3	185	192	1.64	0.39 (0.95)	0.41 (1)
4	990	172	1.84	0.46 (0.98)	0.47 (1)

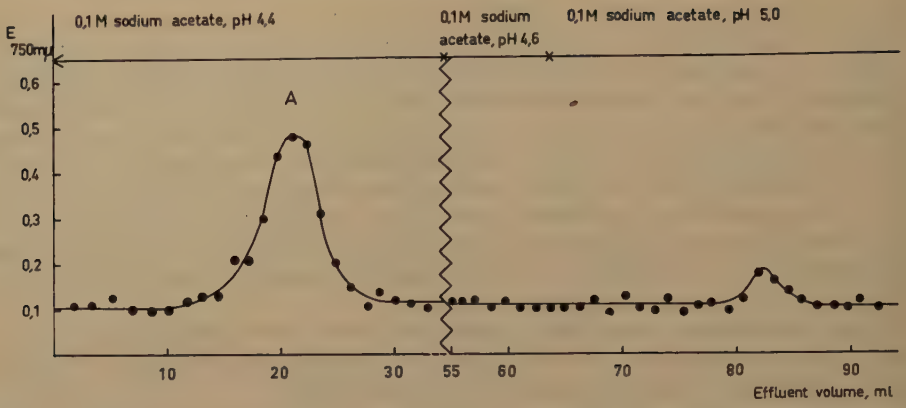

Fig. 11. Appearance of peptide A after incubation of fibrinogen with snake venom enzyme. Chromatography on Dowex-50 × 2 of clot supernatant after 185 minutes' incubation with Reptilase. For further explanation, see text and Table 1.

lumes. N-terminal analysis was made on one of the Reptilase peptide A peaks (Table 1, exp. 4). As could be expected, glutamic acid was identified as the N-terminal. The yield of glutamyl residues, calculated on the amount originally found in fibrinogen, was approximately 50 %.

The experimental procedure in the above experiments was briefly as follows. Coagulation was carried out at pH 6.3–6.4, ionic strength 0.15, and a protein concentration of 0.23 %. Phosphate buffers were used, the concentration of phosphate in the mixture being 0.01 *M*. Sodium chloride was used for adjusting the ionic strength of the solution. 210 mg of fibrinogen were used in each experiment. Reptilase was added to a concentration corresponding to about 0.05 NIH units/ml (coagulation time in this system 10–15 minutes).

The preparation of clot supernatants and chromatography on 2 % cross-linked Dowex-50 (pre-equilibrated with 0.1 *M* sodium formate buffer, pH 3.1) has been described by Blombäck & Vestermark (1958). Before elution of the peptides, the columns had to be washed with 0.1 *M* sodium formate buffer, pH 3.5, for a comparatively long time in order to elute various impurities derived from the enzyme preparation.

In these experiments, the peptides were rechromatographed.

Discussion

In previous investigations, it was found that the release of peptides A and B in a fibrinogen–thrombin system did not run parallel (Bettelheim, 1956; Blombäck & Vestermark, 1958). In the initial phase of transformation only peptide A was released. The latter authors found that at a point where about 40 % of the A peptide had been split off, only about 8 % of peptide B could be demonstrated. In later stages, successively increasing amounts of peptide B were released. These observations have now been confirmed by direct N-terminal determinations in fibrinogen-thrombin systems. The time displacement in the release of the two peptides during conversion of fibrinogen to fibrin results in a diphasic curve, when the glycyl residues which appear in a fibrinogen-thrombin mixture are plotted against time. Since

peptide A contains glutamic acid and/or glutamine as N-terminal residue, the release of peptide A from fibrinogen could also be studied by measuring the release of glutamyl residues from fibrinogen. During the first phase of conversion there was, in fact, a correlation between the appearance of glycyl residues and the disappearance of glutamyl residues. Thus, under different experimental conditions, the glutamyl residues released were equal in number to the glycyl residues that appeared up to a point where about 50–70 % of the glutamyl residues originally present had been split off. However, determination of the glutamyl residues in the later stages, when small amounts were present, may be somewhat unreliable since it has been found that, under certain experimental conditions, the PTH glutamic acid may to a small extent be bound to the protein moiety (Blombäck & Yamashina, 1958).

Thrombin and Reptilase seem to have a remarkably narrow specificity of proteolytic action. The fact that only peptide A is split off by Reptilase stresses the possible importance of the release of this peptide from the fibrinogen for subsequent polymerization. When bovine thrombin is used, the release of peptide A also runs parallel with fibrin formation, whereas peptide B is released at a maximum rate when fibrin formation is near completion.

It was shown by Howell & Holt, as early as 1918, that heparin together with a co-factor in plasma was a potent inhibitor of the coagulation of fibrinogen by thrombin. Heparin together with its co-factor has proved to be not only a strong inhibitor of the thrombin activity as measured in a coagulating system, but also of its proteolytic activity. It is interesting to note that heparin alone also inhibited the reaction. A probable explanation of this finding could be that, in the present system, one of the reagents may have contained traces of heparin co-factor. It has, however, recently been shown that the esterase activity of thrombin, as measured on tosylarginine methyl ester, is not inhibited by heparin alone in concentrations up to 20 units/ml, whereas addition of heparin plus co-factor to this system produces strong inhibition of the thrombin activity (Blombäck & Blombäck, 1958). This finding suggests that our thrombin preparation is not contaminated by the heparin co-factor. As regards the fibrinogen, coagulation studies indicate that preparations of the type used are free from heparin co-factor, since there is no increase in anti-thrombic activity on increasing the fibrinogen concentration in the presence of heparin. It appears likely, therefore, that the inhibition by heparin alone depends on a combination of heparin, which has a strongly negative electric charge, with charged groups on the substrate as well as on the enzyme, thereby hampering the enzyme substrate combination. This mode of action has been suggested following the discovery by Jorpes in 1935–1936 that heparin is a polysulphuric acid ester of mucoitin (*cf.* Jorpes, 1946). The interaction of heparin with various proteins has been demonstrated (Jaques, 1943; Nilsson & Wenckert, 1954).

It has previously been suggested that proteolytic splitting off of a peptide from the fibrinogen molecule is indispensable for subsequent polymerization. Although direct evidence supporting this hypothesis is still lacking, several facts afford indirect evidence of the importance of the proteolytic reaction occurring at the amino end of the polypeptide chains for subsequent polymerization. These facts are: (1) It has so far been impossible to separate the coagulating and the proteolytic activity of thrombin. Thus, the same changes in the N-terminal amino acid pattern are produced by a highly purified thrombin preparation as by a less pure one. (2) Different enzymes which are able to convert fibrinogen to fibrin also split off at least one of the fibrinopeptides. (3) In all fibrins so far obtained from different species and under different

experimental conditions, limited proteolysis has invariably occurred, which implies that in every instance fibrin formation has been preceded by proteolysis (4). Both coagulation and proteolysis are influenced in about the same way by ionic strength, temperature and substrate concentration. The pH optimum for proteolysis is also in fairly good agreement with the pH optimum for initial polymerization (5). Heparin, either alone or in combination with its co-factor, inhibits both coagulation and proteolysis.

SUMMARY

The proteolytic action of thrombin on bovine fibrinogen has been studied. The appearance of N-terminal glycyl residues during transformation of fibrinogen to fibrin has been shown to follow a diphasic curve with respect to time. This is due to the fact that peptide B is released at a slower rate than peptide A in the initial stage of the transformation. The dependence of the proteolytic action of thrombin on pH, ionic strength and temperature, as well as on enzyme and substrate concentrations, has also been studied. It has been shown that heparin, either alone or together with its co-factor in plasma, inhibits the proteolytic action of thrombin.

During the coagulation of bovine fibrinogen with Reptilase, only peptide A is released.

This investigation was carried out at Chemistry Department II, Karolinska Institutet, Stockholm.

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On the complex formation between silver and thiosulphate ions

II. Potentiometric measurements

By Rolf O. Nilsson

With 20 figures in the text

In the present paper the complex formation between silver and thiosulphate ions is studied by the aid of potentiometric measurements in unsaturated and in supersaturated solutions at 25°C with the ionic strength 4 M. Earlier works in this field have been summarized in a previous paper (Nilsson [1]) dealing with solubility investigations on the same system.

Method

Measurements were taken in the form of potentiometric titrations, the concentration of free silver ions $[Ag^+]$ being measured in cell (2) with the aid of silver electrodes as a function of the total thiosulphate concentration. The total silver content was kept constant within each series. To keep the activity coefficients constant sodium perchlorate was added up to the "ionic strength" of 4 M. Thus we have

$$3\,c_{Na_2A} + c_{NaClO_4} + c_{AgClO_4} = 4 \tag{1}$$

in all solutions. The symbol A stands for the S_2O_3 group.

$$-\mathrm{Ag}\,\Big|\,\mathrm{S}\,\Big|\begin{matrix}4\text{ M }NaClO_4\text{ or}\\ 4/3\text{ M }Na_2A\end{matrix}\Big|\,4\text{ M }NaClO_4\,\Big|\,\mathrm{RE}+ \tag{2}$$

The composition of the solution S is defined by (1) and the emf of cell (2) is given by

$$E_m = E_0 - 59.16 \log [Ag^+] \tag{3}$$

In the calculations use was made of

$$E = E_m - E_{m_0} = 59.16 \log \frac{c_{Ag}}{[Ag^+]} = 59.16 \log F \tag{4}$$

E_{m_0} which is the emf of cell (2) when $c_{Na_2A} = 0$ was obtained from measurements in silver perchlorate solutions. E is consequently the emf of the following hypothetical cell which has the same total silver content in the two half-cells.

$$-\mathrm{Ag}\,\Big|\,\mathrm{S}\,\Big|\,4\ \mathrm{M}\ \mathrm{NaClO_4}\,\Big|\begin{matrix} c_{Ag}\ \mathrm{M}\ \mathrm{AgClO_4} \\ (4-c_{Ag})\ \mathrm{M}\ \mathrm{NaClO_4}\end{matrix}\Big|\,\mathrm{Ag}+ \qquad (5)$$

No correction was made for the diffusion potentials as the ionic mobilities were unknown.

Experimental

Reagents and apparatus

The *silver electrodes* and the *4/3 M* $Na_2S_2O_3$ and *4 M* $NaClO_4$ stock solutions were prepared as described in a previous paper (Nilsson [1]).

4 M $AgClO_4$ was made according to Leden [2].

The potentiometer, the galvanometer, and the *reference electrodes* used, were the same as described before (Ref. [3]) and the *nitrogen* was purified in the same way as described there.

Procedure

The titration series were carried out as follows. A solution of the composition c_{Ag} M $AgClO_4$, $(4-c_{Ag})$ M $NaClO_4$ was added from a burette to a solution containing c_{Ag} M $AgClO_4$, c_A M Na_2A, and $(4-c_{Ag}-3\,c_A)$ M $NaClO_4$ and the emf of cell (2) was measured after each addition. At the end of the titration series precipitation occurred. As could be shown by X-ray analysis the precipitated crystals were $NaAgA \cdot H_2O$ in all series. To check the reproducibility of the electrodes and to control that no precipitation had started in the solutions always at least two series were run with the same c_{Ag}. The emfs could be reproduced within 0.2 mV. Nitrogen was bubbled through the solutions while the measurements were taken. It was possible to supersaturate the solutions in all of the series and in a few cases the ratio $c_{Ag} : c_A$ was as high as 0.98. (It was found, that some of the electrodes used initiated the precipitation at an early stage. These electrodes were exchanged against ones which did not behave in such a way during the measurements. Equilibrium was almost instantly reached on adding $AgClO_4$ solution and, as a rule, the potentials obtained were steady. In the most supersatured solutions a slow drift of the emfs were sometimes observed, probably caused by the beginning precipitation of $NaAgA \cdot H_2O$. These values were not used in the calculations, however. In some cases a small drop of 2 M NaOH was added to the solution before the titration. It could be stated that the emfs remained the same as if no NaOH would have been added. This shows that $HS_2O_3^-$ (with $K_s = 10^{-2}$) and silver hydroxide complexes have no influence upon the measurements. To get E_{m_0} required for the calculation of E (equation (4)), cell (2) was measured with $c_A = 0$. It was found, that the electrodes followed Nernst's law for $c_{Ag} \geq 2$ mM (*cf* Table 7, Leden and Nilsson [4]). At $c_{Ag} = 1$ mM equilibrium was attained only after several minutes.

Eleven titration series were made with $c_{Ag} = 1$, 2, 5, 10, 20, 30, 40, 50, 65, 80, and 100 mM. This large number of titrations was necessary, because the concentration of free thiosulphate ions which could not be measured had to be evaluated by a graphical integration method. Many values of c_{Ag} are then required to give acceptable results. The experimental values are given in Table 1.

Table 1. Corresponding values of c_A and E. About one third of the experimental points are given.

c_{Ag} = 100 mM		c_{Ag} = 80 mM		c_{Ag} = 65 mM		c_{Ag} = 50 mM	
c_A mM	E mV	c_A mM	E mV	c_A mM	E mV	c_A mM	E mV
149.3	571.7	94.6	468.6	96.0	550.4	73.9	537.4
154.8	579.6	96.3	479.3	99.1	559.0	77.4	547.2
160.5	586.8	100.0	500.5	105.6	569.4	81.3	556.1
166.7	593.6	104.0	519.5	111.2	577.9	85.6	564.4
171.1	600.3	108.4	535.0	117.3	586.0	92.9	579.2
180.6	607.7	113.1	584.4	122.4	591.8	101.6	587.5
188.4	613.2	123.9	570.5	128.0	597.5	108.3	595.0
197.0	619.0	136.9	588.8	134.1	602.9	130.0	614.7
206.4	624.9	153.0	604.9	150.9	616.2	154.8	630.8
216.7	630.8	167.7	612.2	162.5	623.7	171.0	639.6
240.7	642.3	185.7	627.7	176.0	631.5	191.2	649.3
270.8	654.3	200.0	635.4	201.1	644.0	216.7	660.2
309.5	667.0	216.7	643.3	234.7	657.8	232.0	666.6
361.1	681.2	236.4	651.5	248.5	662.8	267.0	678.8
406.2	692.8	260.0	660.4	281.7	674.1	333.0	698.3
464.0	705.4	298.9	669.9			417.0	717.4
542.0	719.9	325.0	680.6			500.0	732.6
650.0	726.7	346.7	686.5			583.0	745.1

c_{Ag} = 40 mM		c_{Ag} = 30 mM		c_{Ag} = 20 mM		c_{Ag} = 10 mM	
c_A mM	E mV	c_A mM	E mV	c_A mM	E mV	c_A mM	E mV
46.4	433.1	40.6	490.3	25.0	441.6	10.2	330
47.3	442.6	42.4	502.0	26.0	457.6	10.8	352.6
48.1	452.3	44.3	512.0	27.1	470.9	11.2	365.1
49.0	462.4	46.4	521.8	28.3	482.7	11.6	370.1
50.0	472.2	48.8	530.5	30.9	502.1	12.0	397.4
51.0	480.8	54.2	546.3	32.5	510.7	12.5	413.4
52.0	489.2	60.9	561.1	36.1	526.2	13.0	427.0
54.2	503.6	65.0	568.1	40.6	540.5	13.5	439.2
56.5	516.2	73.6	581.2	44.8	551.3	14.1	449.8
61.9	536.8	84.8	594.4	50.0	562.0	14.8	459.2
65.0	545.7	92.9	602.4	56.5	573.1	15.5	467.7
72.2	561.7	108.3	615.7	61.9	581.0	16.3	475.2
81.2	576.7	114.7	620.7	68.4	589.3	18.1	489.5
92.8	591.4	121.9	625.7	76.5	598.4	20.3	503.2
100.0	598.7	130.0	631.3	86.7	608.4	23.2	516.8
113.0	610.3			106.1	625.6	26.0	527.4
123.8	618.5			133.0	644.0	31.7	544.4
136.8	627.3			166.2	661.4	37.1	557.3
152.9	636.8			203.0	677.3	41.7	567.1
173.3	647.4			272.3	699.2	51.3	584.5
				410.0	729.6	60.6	596.0
				517.0	746.2	69.7	607.5
				632.1	760.3	95.3	633.1
				967.0	790.0	111.1	644.7
						167.0	674.6
						202.3	687.5
						267.0	706.8
						333.2	722.2
						450.1	743.2
						596.3	761.7

Table 1 (*continued*)

$c_{Ag}=5$ mM		$c_{Ag}=2$ mM		$c_{Ag}=1$ mM	
c_A mM	E mV	c_A mM	E mV	c_A mM	E mV
5.08	303.4	2.17	286.6	1.08	259.7
5.24	313.2	2.24	297.4	1.12	268.9
5.42	323.9	2.32	309.5	1.16	279.3
5.60	335.7	2.41	323.3	1.20	291.1
5.80	349.3	2.50	337.0	1.25	303.5
6.02	364.9	2.60	349.4	1.30	314.7
6.25	379.9	2.83	369.3	1.41	333.7
6.50	393.2	3.25	392.7	1.55	349.4
8.12	439.5	3.82	412.2	1.71	363.0
8.55	446.4	4.33	425.1	2.03	381.7
9.56	459.6	4.82	434.5	2.41	397.5
10.8	472.8	5.65	448.2	2.95	414.7
13.0	489.7	6.84	463.8	3.61	431.0
14.8	500.7	8.11	477.6	4.33	445.5
17.1	512.7	9.24	487.9		
19.1	521.8	10.4	497.1		
21.7	531.7	12.3	509.8		
25.6	546.6	14.5	521.3		
30.3	559.4	20.8	545.5		
39.2	578.1	30.3	571.8		
47.6	592.2	47.6	599.2		
55.5	603.1	70.2	622.7		
70.1	619.1	116.0	653.6		
83.3	629.5	167.1	674.4		
106.0	648.8	229.2	696.7		
133.1	661.7	404.1	734.3		
167.2	676.4				
210.1	692.6				
266.3	708.7				
317.0	720.1				
402.0	733.2				
512.1	752.1				

Determination of the mononuclear complexes

If the complex formation is written

$$m\,Ag^+ + n\,A^{2-} \rightleftarrows Ag_m A_n^{(2n-m)-} \tag{6}$$

then the system is defined by the complexity constants

$$\beta_{m,n} = \frac{[Ag_m A_n]}{[Ag^+]^m [A]^n} \tag{7}$$

The total silver concentration is given by

$$c_{Ag} = \sum_{m=1} \sum_{n=0} m\,[Ag_m A_n] = [Ag^+] + \sum_{m=1} \sum_{n=1} m\,\beta_{m,n}\,[Ag^+]^m [A]^n \tag{8}$$

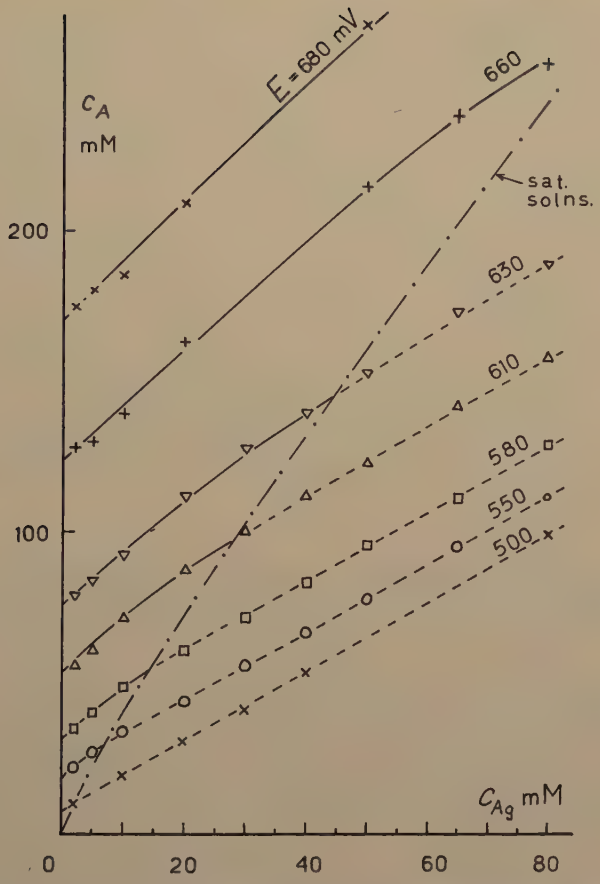

Fig. 1. Extrapolation of the total concentration of thiosulphate *vs.* total silver content to $c_{Ag} = 0$ at constant E. The drawn curves (broken for supersaturated solutions) are calculated according to (4), (57), and (58) from the constants in Table 5.

and the total thiosulphate content by

$$c_A = \sum_{m=0} \sum_{n=1} n [Ag_m A_n] = [A] + \sum_{m=1} \sum_{n=1} n \beta_{m,n} [Ag^+]^m [A]^n \qquad (9)$$

The ligand number, $\bar{n}$, is defined by

$$\bar{n} = \frac{c_A - [A]}{c_{Ag}} \qquad (10)$$

The potentiometric measurements give $[Ag^+]$ as a function of c_A with c_{Ag} constant in each series. From equation (8) it can be seen that $[Ag^+]$ as well as [A] have to be known for the determination of $\beta_{m,n}$. If one however extrapolates $c_{Ag} \to 0$, [A] approaches c_A at the same time as the polynuclear complexes disappear. Consequently, already at this point such an extrapolation gives

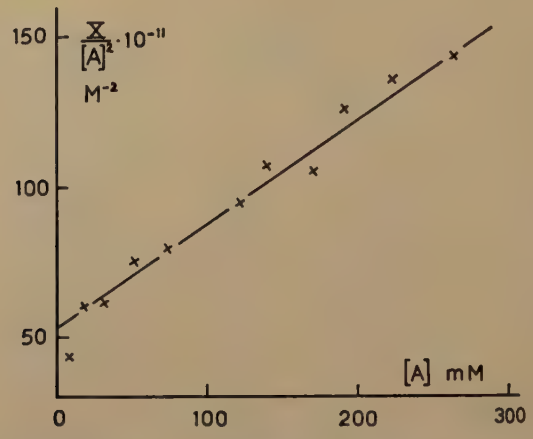

Fig. 2. Graphical evaluation of the equilibrium constants for the mononuclear complexes (see (11)).

the constants for the mononuclear complexes. The extrapolation is made according to Fronæus [5] i.e. c_A is dravn as a function of E at constant c_{Ag}. The curves are then cut for a series of values of E and c_A is drawn against c_{Ag} with E as a parameter (Fig. 1). Extrapolation to $c_{Ag}=0$ gives [A] for the E value in question. If only mononuclear complexes were present, the experimental points would lie on a straight line with the slope $\bar{n}$ (*cf.* (4), (8), and (10)). In the silver-thiosulphate system this is not the case, as the mononuclear complexes dominate only at low values of c_{Ag} and at high values of c_A i.e. where the concentration of the polynuclear species in negligible. Corresponding values of E, from which $X=\left(\frac{c_{Ag}}{[Ag^+]}\right)_{c_{Ag}=0}$ can be calculated, and values for [A] are in this way obtained. From (8) we get

$$X/[A]^2 = 1/[A]^2 + \frac{\beta_{1,1}}{[A]} + \beta_{1,2} + \beta_{1,3}[A] + \beta_{1,4}[A]^2 + \cdots \qquad (11)$$

which is plotted against [A] in Fig. 2. The points can be represented by a straight line with the slope $\beta_{1,3}=3.3\cdot 10^{13}$ and the origo ordinate $\beta_{1,2}=5.3\cdot 10^{12}$. Mononuclear complexes richer in thiosulphate (AgA_4, AgA_5 etc.) should have given experimental points above the straight line at high values of [A] which is not the case. The complex AgA should have given a rising curve with decreasing values of [A] contrary to what was found in the range investigated.

$\beta_{1,2}$ can be recalculated to $k_{1,1}=\beta_{1,2}k_{1,-1}=0.30$ (*cf.* (11) in Ref. [1]). From the solubility measurements $K_1=0.30$ was evaluated and the constants $k_{m,1}$ $(m>1)$ are thus zero, or near zero. In the same way $\beta_{1,3}$ gives $k_{1,2}=\beta_{1,2}k_{1,-1}=1.9$ which is lower than the value $K_2=8$, obtained from the solubility measurements and this means, that Ag_mA_{m+2} $(m>1)$ ions are also present.

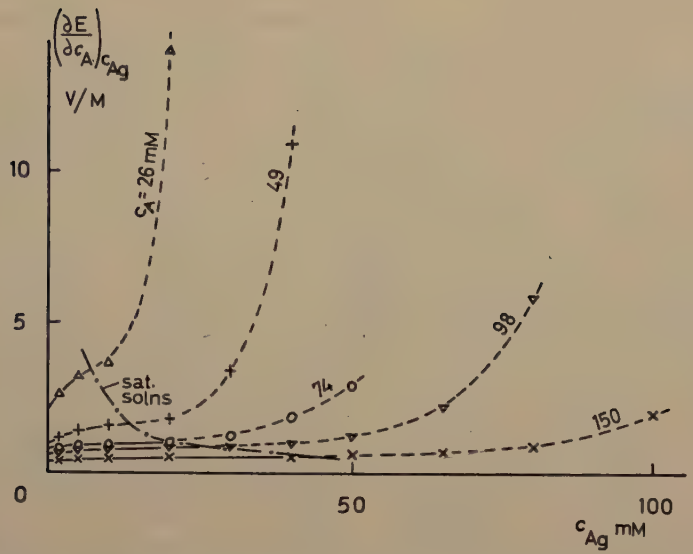

Fig. 3. Evaluation of the free thiosulphate concentration according to equation (12). The curves (broken for supersaturated solutions) are obtained graphically from a $E(c_A)c_{Ag}$ diagram, calculated according to (4), (57), and (58) from the constants in Table 5.

Determination of the free thiosulphate ion concentration

To determine the complexity constants of the polynuclear ions it is necessary to know corresponding values of $[Ag^+]$ and [A]. [A] cannot be measured, as no suitable electrode is known, but it can be evaluated from the measurements by graphical integration according to a formula by Hedström [6]. With the symbols used here the equation is written as

$$\log \frac{[A]}{c_A} = \left[\int_0^{c_{Ag}} \left(\frac{\partial \log \frac{[Ag^+]}{c_{Ag}}}{\partial c_A}\right)_{c_{Ag}} dc_{Ag}\right]_{c_A} = -\frac{F}{RT}\left[\int_0^{c_{Ag}} \left(\frac{\partial E}{\partial c_A}\right)_{c_{Ag}} dc_{Ag}\right]_{c_A} \qquad (12)$$

To find [A] in the point c_{Ag}, c_A the quantities $\left(\frac{\partial E}{\partial c_A}\right)_{c_{Ag}}$ are measured from an $E(c_A)c_{Ag}$ diagram and plotted against c_{Ag}. The area under a c_A curve from $c_{Ag}=0$ to c_{Ag} is then equal to $-\frac{RT}{F}\log\frac{[A]}{c_A}$ from which [A] can be calculated. Some of the [A] values thus obtained are given in Table 2.

It is seen that [A] drops down to less than 1 % of c_A, when c_A approaches c_{Ag}. Fig. 3 shows a few examples of $\left(\frac{\partial E}{\partial c_A}\right)_{c_{Ag}}$ *vs.* c_{Ag} curves. To obtain sufficient accuracy of the [A] values it is obviously necessary to make measurements at many values of c_{Ag} in order to obtain enough experimental points on each

Table 2. Corresponding values of c_A, [A] (obtained according to Hedström [6]), [Ag^+] (obtained from $E\,(c_A)_{c_{Ag}}$ curves) and $\bar{n}$.

c_{Ag} = 100 mM				c_{Ag} = 80 mM				c_{Ag} = 65 mM			
c_A mM	[A] mM	[Ag^+] 10^{13} M	$\bar{n}$	c_A mM	[A] mM	[Ag^+] 10^{13} M	$\bar{n}$	c_A mM	[A] mM	[Ag^+] 10^{13} M	$\bar{n}$
150	8.3	236	1.42	96	0.55	6960	1.19	96	5.8	327	1.39
160	13.0	129	1.47	100	1.00	2810	1.24	100	7.7	232	1.42
170	18.9	80.0	1.51	105	2.46	1160	1.28	105	10.1	165	1.46
180	24.1	55.6	1.56	115	5.59	364	1.36	115	14.9	92.0	1.54
190	29.3	42.0	1.61	130	12.1	127	1.47	130	23.2	48.8	1.65
200	34.7	32.3	1.65	150	22.5	53.7	1.60	140	29.6	35.2	1.69
225	51.2	18.5	1.74	170	35.9	29.0	1.68	150	36.0	26.2	1.75
250	66.4	12.0	1.84	190	47.6	18.1	1.78	170	52.0	16.0	1.82
300	99.3	6.02	2.01	200	55.5	14.7	1.81	180	58.0	12.9	1.88
350	135	3.49	2.15	225	71.6	9.44	1.92	200	71.5	8.77	1.97
400	176	2.01	2.24	250	93.2	6.45	1.96	225	90.5	5.80	2.06
500	271	0.926	2.29	300	127	3.39	2.16	250	110	4.06	2.15
600	356	0.474	2.44	350	167	1.94	2.29	300	152	2.13	2.28

c_{Ag} = 50 mM				c_{Ag} = 40 mM				c_{Ag} = 30 mM			
c_A mM	[A] mM	[Ag^+] 10^{13} M	$\bar{n}$	c_A mM	[A] mM	[Ag^+] 10^{13} M	$\bar{n}$	c_A mM	[A] mM	[Ag^+] 10^{13} M	$\bar{n}$
74	4.8	406	1.38	47	0.30	15,100	1.17	41	1.70	1420	1.31
83	9.3	179	1.47	50	0.65	4,220	1.24	42	1.95	1110	1.33
96	16.3	77.5	1.60	52	1.20	2,190	1.27	43	2.34	877	1.36
100	19.1	64.5	1.62	57	2.84	698	1.36	44	2.72	711	1.38
115	28.0	33.6	1.74	60	4.24	425	1.40	47	4.68	418	1.41
130	37.9	20.6	1.84	74	11.6	115	1.56	50	6.35	280	1.46
140	44.9	15.7	1.90	83	17.7	65.7	1.63	52	7.44	220	1.49
180	77.8	6.43	2.04	88	20.5	51.0	1.62	54	8.36	177	1.52
200	92.7	4.59	2.15	100	29.0	30.8	1.78	60	12.0	107	1.60
225	114	3.07	2.23	110	35.7	21.6	1.86	74	21.6	44.7	1.75
250	136	2.14	2.28	150	66.8	7.3	2.08	88	31.5	24.0	1.89
300	181	1.14	2.38					100	41.1	15.4	1.96
350	225	0.67	2.50					120	57.4	8.6	2.08

curve of constant c_A in Fig. 3. The errors in [A] are bigger at the lower silver concentrations than at the higher ones, as the relative differences between the c_{Ag} values chosen here are bigger at low silver contents. It is also obvious that the errors at a certain c_A value become largest when the ratio c_A/c_{Ag} approaches 1, as $(\partial E/\partial c_A)_{c_{Ag}}$ here rises rapidly. At the end of some titration series (*cf.* Fig. 20) $(\partial E/\partial c_A)_{c_{Ag}}$ drops after reaching its maximum value and this means, that if one made measurements at *very* many c_{Ag} values one should get a maximum in Fig. 3, if curves were plotted for the lowest values of c_A/c_{Ag}.

Table 2 (*continued*)

$c_{Ag}=20$ mM				$c_{Ag}=10$ mM				$c_{Ag}=5$ mM			
c_A mM	[A] mM	$[Ag^+]$ 10^{13} M	$\bar{n}$	c_A mM	[A] mM	$[Ag^+]$ 10^{13} M	$\bar{n}$	c_A mM	[A] mM	$[Ag^+]$ 10^{13} M	$\bar{n}$
25	0.32	5540	1.23	11	0.079	89,500	1.09	10	1.9	692	1.62
27	0.96	2260	1.30	12	0.092	21,000	1.19	15	6.0	166	1.80
30	2.33	830	1.38	14	0.745	2,720	1.33	25	14.3	30.8	2.14
34	4.55	359	1.47	15	1.35	1,580	1.37	34	23.7	12.8	2.06
38	6.94	201	1.56	16	1.80	1,040	1.42	44	33.2	6.02	2.16
42	8.96	128	1.65	21	4.38	274	1.66	74	62.7	1.48	2.26
44	10.2	104	1.69	25	7.15	142	1.78	100	89.0	0.678	2.20
50	15.0	64.0	1.75	30	11.7	75.2	1.83	200	188	0.114	2.40
74	34.1	17.1	2.00	44	23.4	21.9	2.06	300	287	0.043	2.54
100	58.4	6.48	2.08	52	31.1	12.9	2.09				
150	103	1.82	2.33	74	51.6	4.37	2.24				
200	152	0.763	2.42	150	126	0.544	2.36				
300	249	0.232	2.55	300	274	0.083	2.56				

$c_{Ag}=2$ mM			
c_A mM	[A] mM	$[Ag^+]$ 10^{13} M	$\bar{n}$
10	5.9	90.7	2.20
15	10.8	28.3	2.13
25	20.6	7.50	2.20
34	29.8	3.51	2.10
74	69.6	0.528	2.20
105	101	0.231	2.10
200	195	0.0473	2.40
300	295	0.0175	2.45

The functions Z_0 and Z_1

When corresponding values of $[Ag^+]$ and [A] had been found the next step was to find a reaction scheme which could explain these measurements. In such cases it is convenient to study the spacing between curves when some suitable function is drawn in a logarithmic scale (Sillén [7]). Such a function is $\bar{n}$ (log [A])$_{c_{Ag}}$, but as the error in [A] appears in a very unfavorable way in this diagram, no certain conclusions could be drawn from it. A somewhat more suitable function is $\log F = \log \frac{c_{Ag}}{[Ag^+]}$ *vs.* log [A] with c_{Ag} as parameter, which is shown in Fig. 4, but as at low values of c_A the [A] values are relatively uncertain, especially at low values of c_{Ag}, no certain conclusions could be drawn from this diagram either. As $[Ag^+]$ is the only quantity which is known at high c_{Ag}/c_A ratios it seemed reasonable to try the following function

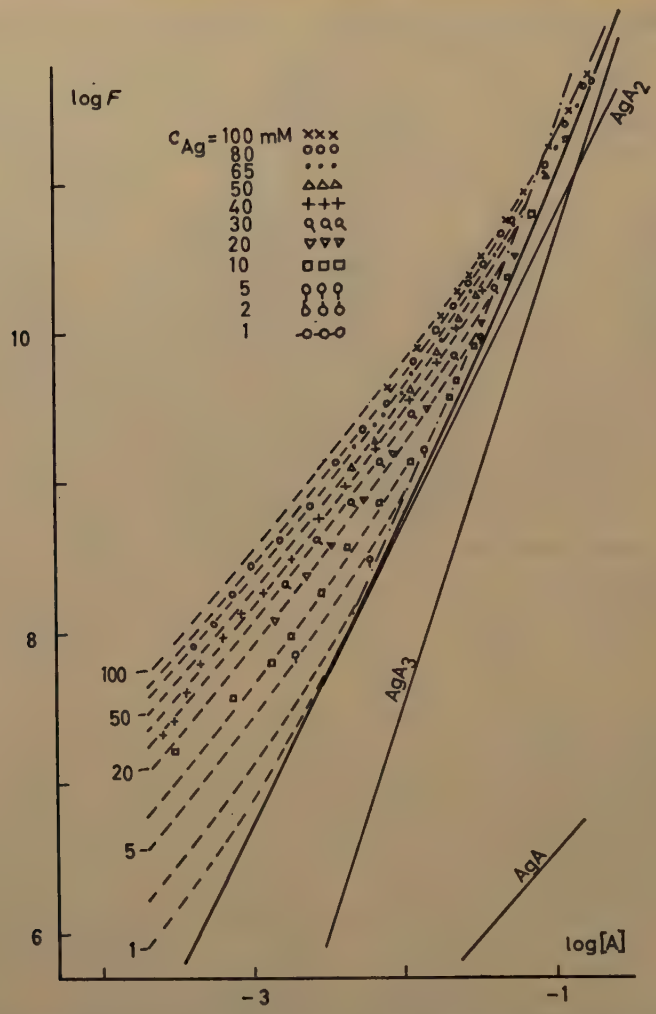

Fig. 4. log F (defined by (4)) as a function of the logarithm of the free thiosulphate concentration at constant c_{Ag}. The [A] values evaluated according to Hedström [6]. The thin curves (broken for supersaturated solutions) are calculated according to (28) and (57) from the constants in Table 5. The thick curve represents the sum of the mononuclear complexes, each of which is given as a straight line.

$$Z_0 = \frac{c_{Ag} - [Ag^+]}{c_A} \approx \frac{c_{Ag}}{c_A} \tag{13}$$

In Fig. 5 this function is plotted against log $[Ag^+]$.

The experimental points are plotted at constant c_{Ag} and not at constant c_A which is usually the case (*cf.* Ref. [7]), as the measurements were carried out at constant c_{Ag} values. In Fig. 6 log c_{Ag} *vs.* log $[Ag^+]$ is shown for five values of Z_0. The slope of the lines

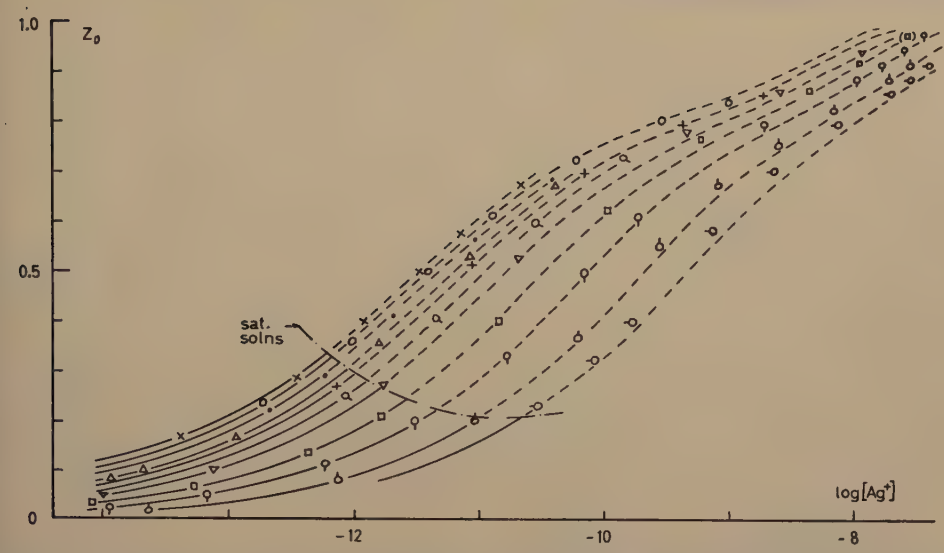

Fig. 5. Z_0 (defined by (13)) *vs.* the logarithm of the free silver ion concentration at constant c_{Ag}. The symbols are the same as in Fig. 4. The curves (broken for supersaturated solutions) are calculated according to (15) with the constants in Table 5.

$$\left(\frac{\Delta \log c_{Ag}}{\Delta \log [Ag^+]}\right)_{Z_0} = R \qquad (14)$$

is not constant but decreases from -0.82 to -1.50 although it remains in the neighbourhood of -1 at $Z_0 = 0.3 - 0.7$.

The following conditions must hold if the curves in Fig. 5 are to be parallel (*i.e.* for the lines in Fig. 6 to have the same slope). The proof is made in analogy to a derivation by Sillén [7]. If any complex Ag_mA_n exists, then

$$Z_0 = \frac{\Sigma\, m\, \beta_{m,n} [Ag^+]^m [A]^{n-1}}{1 + \Sigma\, n\, \beta_{m,n} [Ag^+]^m [A]^{n-1}} \qquad (15)$$

but if the curves in Figure 5 are parallel, Z_0 must be a function of

$$[Ag^+]^{-R} c_{Ag} = [Ag^+]^{-R} ([Ag^+] + \Sigma\, m\, \beta_{m,n} [Ag^+]^m [A]^n) \approx \Sigma\, m\, \beta_{m,n} [Ag^+]^{m-R} [A]^n$$

From these expression we may draw the following conclusions

1) If [A] is *not negligible* in comparison with c_A all terms in (15) must be powers of the same variable $[Ag^+]^{\frac{m-R}{n}} [A]$. Thus $\frac{m}{n-1} = \frac{m-R}{n}$ or

$$m = -R\,(n-1) \qquad (16)$$

The equation for a series of complexes $Ag_r (Ag_tA)_i$ or $A_s (Ag_tA)_i$ in a m, n diagram is (Ref. [7])

$$m = t\,n + r = t\,(n - s) \qquad (17)$$

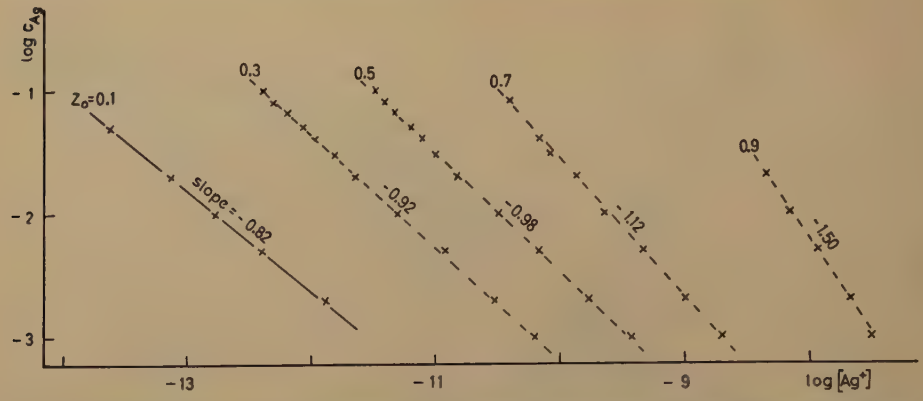

Fig. 6. Determination of curve spacing. The most representative straight lines (broken for supersaturated solutions) are shown.

If (16) is compared with (17) we find that

$$R = r = -t \quad \text{and} \quad s = 1. \tag{18}$$

2) If [A] is *negligible* in comparison with c_A, (15) can be multiplied with $[Ag^+]^{-R}[A]$ so that all terms contain $[Ag^+]^{m-R}[A]^n$. As we also need them as powers of the same variable $[Ag^+]^t[A]$ we get

$$\frac{m-R}{n} = t \quad \text{or} \quad m - R = nt \tag{19}$$

which compared with (17) gives

$$R = r \tag{20}$$

The conditions for parallelism in Fig. 5 where the curves were drawn at constant c_{Ag} are thus the same, as if they were drawn at constant c_A (*cf.* Ref. [7]) depending upon that $[Ag^+]$ is negligible besides c_{Ag}.

The slopes in Figure 6 are ≈ -1 for $Z_0 < 0.7$, i.e. in the range where [A] cannot be neglected in comparison with c_A and this would according to (18) mean that we have complexes with the composition $A(AgA)_m$. It was shown in a previous section that AgA_3, which does not fit into this series, is also formed in the solutions, and the system is consequently at first treated under the assumption, that the deviation of the slopes from the value -1 is caused by AgA_3 and that, besides this complex only groups of the composition $A(AgA)_m$ are formed.

If

$$u = [Ag^+][A] \tag{21}$$

the following expressions for c_{Ag}, c_A and Z_0 are obtained.

$$c_{Ag} = [Ag^+] + \beta_{1,3}\, u\, [A]^2 + [A] \sum m\, \beta_{m,m+1}\, u^m \tag{22}$$

$$c_A = [A] + 3\, \beta_{1,3}\, u\, [A]^2 + [A] \sum (m+1)\, \beta_{m,m+1}\, u^m \tag{23}$$

$$Z_0 = \frac{\beta_{1,3}\, u\, [A]^2 + [A] \sum m\, \beta_{m,m+1}\, u^m}{[A] + 3\, \beta_{1,3}\, u\, [A]^2 + [A] \sum (m+1)\, \beta_{m,m+1}\, u^m} \tag{24}$$

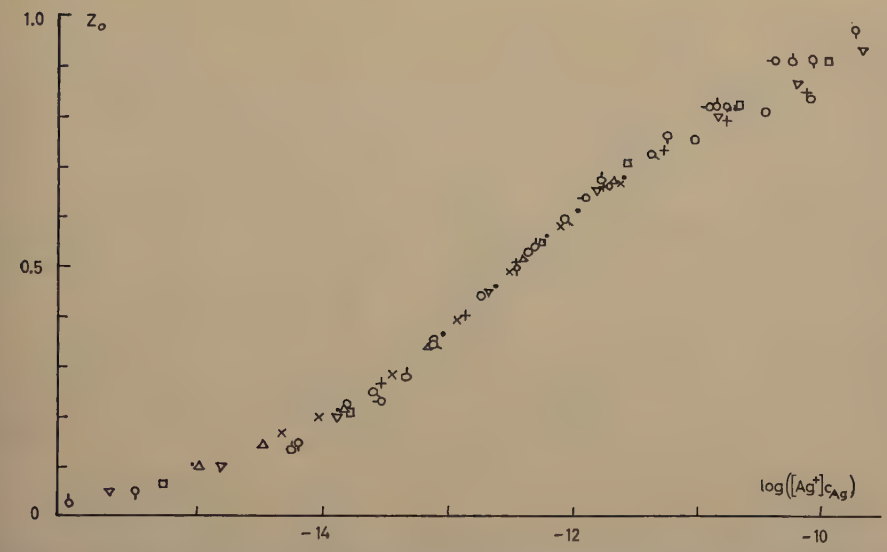

Fig. 7. Z_0 (defined by (13)) drawn against log $[Ag^+]\,c_{Ag}$. The symbols are the same as in Fig. 4.

Z_0 is a function of u only if $[AgA_3]$ can be neglected, but as u is not a measured quantity, Z_0 in Fig. 7 has been drawn against $[Ag^+]\,c_{Ag}$ instead (if Z_0 is kept constant, then u = constant and $c_{Ag} - [Ag^+] \approx c_{Ag} = [A] \cdot \text{const.} = u \cdot [Ag^+]^{-1} \cdot \text{constant}$). The points belonging to different c_{Ag} coincide at $Z_0 \approx 0.5 - 0.6$ but below and above this range there is a significant spreading. To examine the influence of AgA_3 a new function

$$Z_1 = \frac{c_{Ag} - [Ag^+] - \beta_{1,3}\, u\, [A]^2}{c_A - 3\,\beta_{1,3}\, u\, [A]^2} \tag{25}$$

with $\beta_{1,3} = 3.3 \cdot 10^{13}$ is plotted *vs.* log $[Ag^+]$ ($c_{Ag} - \beta_{1,3}\, u\, [A]^2$) in Figure 8. In this diagram the experimental points seem to lie on the same curve within the experimental errors in the range $Z_1 = 0$–0.7, but for $Z_1 > 0{,}7$ there is again a significant spreading. Ignoring this spreading it was tried to explain the curve with the aid of two constants. The first of them, $\beta_{1,2} = 0.53 \cdot 10^{13}$ which is the complexity constant for AgA_2, the first member in the $A\,(AgA)_m$ series, is already known. The second of them, c, is the equilibrium constant for addition of a new link.

$$c = \frac{[A\,(AgA)_{m+1}]}{[A\,(AgA)_m]\,[Ag^+]\,[A]} \qquad (m \geq 1) \tag{26}$$

Expressions were derived for c_{Ag}, c_A and Z_1 and the value of c, best describing the experimental values in Fig. 8 was found to be $c = 0.35 \cdot 10^{13}$.

To check this result against the solubility measurements (Ref. [1]), for which the complexes in $A\,(AgA)_m$ all appear in the constant $K_1 = \sum_{m=1} m\, k_{m,1}$ the complexity constants were recalculated to $k_{m,1}$ according to

$$k_{m,1} = \beta_{m,m+1}\, k_{1,-1}^m = \beta_{1,2}\, c^{-1}\, (c\, k_{1,-1})^m \tag{27}$$

In this way $K_1 = 0.300 + 0.122 + 0.036 + 0.010 + 0.002 + 0.001 + \cdots = 0.47$ was obtained which

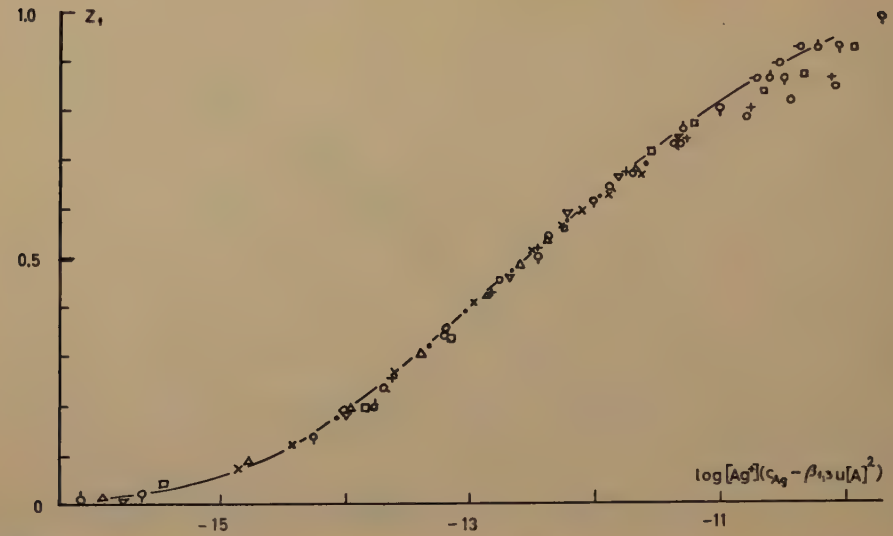

Fig. 8. Z_1 (defined by (25)) *vs.* $\log [Ag^+]$ ($c_{Ag} - [AgA_3]$). Symbols as in Fig. 4. The points have been plotted with $\beta_{1,3} = 3.3 \cdot 10^{13}$. The drawn curve is calculated with $\beta_{1,3} = 5.3 \cdot 10^{12}$ and $c = 3.5 \cdot 10^{13}$ (*cf.* (26)).

is much higher than $K_1 = 0.30$ evaluated from the solubility measurements. From this, one must draw the conclusion that *it is not possible* to explain the potentiometric measurements by AgA_3 and a $A(AgA)_m$ series in spite of the fact that the points in Fig. 8 lie on the same curve over a wide range.

It may be observed that *if the precipitation had occurred at* $Z_1 \approx 0.7$ and *if no solubility measurements had been made* the conclusion from Fig. 8 must have had been, that the series $A(AgA)_m$ was present. When Z_0 approaches 1 the slope in Fig. 6 decreases. At the same time [A] also decreases and becomes of the order of only 1 % of c_A at $Z_0 = 0.95$. This means according to (20) that the slope $R = r$ and that we should have complexes $Ag_r(Ag_tA)_m$ where $r < -1.5$. This is in accordance with the result obtained in the next section, i.e. that we have complexes of the form $A_2(AgA)_m$ or $Ag_{-2}(AgA)_m$.

The F functions

As it was not possible to determine the composition of the complexes from the spreading of the Z_0 curves in Fig. 5, the problem was instead attacked in the following way. From (8) it follows that, if [A] is held constant, c_{Ag} and also the measured quantity

$$F = \frac{c_{Ag}}{[Ag^+]} = 1 + \Sigma\, m\, \beta_{m,n} [Ag^+]^{m-1} [A]^n \qquad (28)$$

are functions of $[Ag^+]$ only. If now $\log F$ is drawn against $\log [Ag^+]$ the slope of the curve for a certain value of [A] would be $m-1$, if a single complex

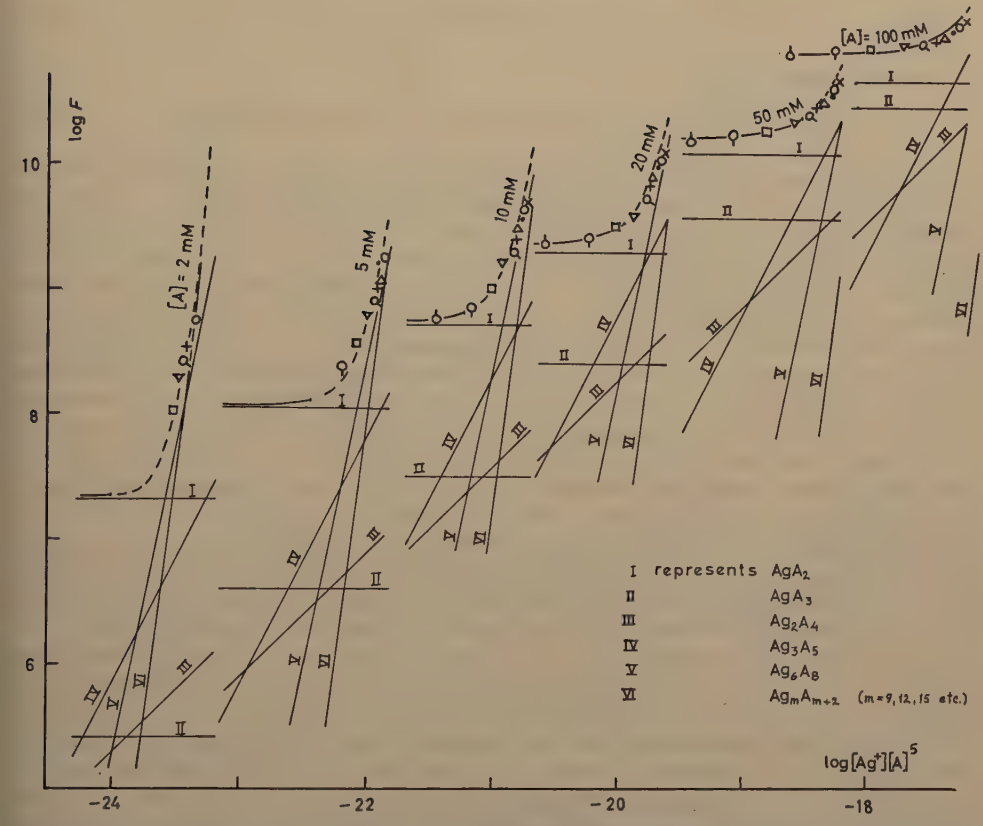

Fig. 9. log F (defined by (5)) *vs.* (log $[Ag^+]\,[A]^5)_{[A]}$. Symbols as in Fig. 4. The calculated curves are broken for supersaturated solutions. The contribution to F by a single complex Ag_mA_n at a certain value of $[Ag^+]\,[A]^5$ can be read off from the thin lines (*cf.* (30)), which are drawn from the constants in Table 5.

Ag_mA_n was present. From a log F (log $[A])_{c_{Ag}}$ diagram (Fig. 4) values of F were read off for the selected values of [A] and then log F (log $[Ag^+]\,[A]^5)_{[A]}$ was plotted in Fig. 9. The abscissa log $[Ag^+]\,[A]^5$ instead of log $[Ag^+]$ was chosen in order to separate the curves better. F is accordingly written

$$F = 1 + \Sigma\, m\beta_{m,n}\,([Ag^+]\,[A]^5)^{m-1}\,[A]^{n-5(m-1)} \qquad (29)$$

From diagram 9 can be seen that the experimental curve at a constant [A] value tends towards a horizontal azymtote when c_{Ag} drops and this means, that the percentage amounts of mononuclear complexes (with the slope $m-1=0$) are increasing. When c_{Ag} rises the curves seem to have a slope about 5, which means that the highest complex in the range covered in the diagram contains 6 silver atoms.

In order to find out which complexes are present, the diagram was examined in the following way. The contribution to F by a single complex Ag_mA_n is

$$F_{m,n} = m\,\beta_{m,n}\,([Ag^+]\,[A]^5)^{m-1}\,[A]^{n-5(m-1)} \tag{30}$$

or in logarithmic form

$$\log F_{m,n} = \log m\,\beta_{m,n}\,([Ag^+]\,[A]^5)^{m-1} + [n-5(m-1)]\,\log [A] \tag{31}$$

On a transparent paper six straight lines, corresponding to the chosen values of [A], 2, 5, 10, 20, 50, and 100 mM, are drawn with the slopes $m-1$ and the vertical spacings $[n-5(m-1)]\,\log C$, where C is the ratio between every two of the consequent values of [A] *i.e.* in this case 2.5 or 2. The value of n on the first transparent paper is 1, on the next 2 and so on, up to 3 m. Such diagrams were prepared for $m=1$, 2, 3 etc. The transparent papers were now moved over the log F (log $[Ag^+]\,[A]^5)_{[A]}$ diagram and it was hereby found that $F_{m,n}$ gave a strong contribution to all six experimental curves in the diagram only when the value of n was $m+2$. This was valid for all values of m except for $m=1$ when $n=2$ (corresponding to AgA_2) gave a stronger contribution to all of the curves than did $n=3$. From this follows that it is probable that all the polynuclear complexes belong to the series Ag_mA_{m+2}. Furthermore it was not necessary to use all values of m in this series, especially not $m=4$ and 5. The lines drawn in Fig. 9 correspond to AgA_2, AgA_3, Ag_2A_4, Ag_3A_5, Ag_6A_8, and $\Sigma\,Ag_{3z}A_{3z+2}$ $(z \geqslant 3)$ and the experimental curves seem to be satisfied by these complexes. The lines representing the mononuclear complexes are drawn with the constants determined in an earlier section and fit well into the diagram. The lines corresponding to the polynuclear complexes are drawn with constants (see Table 5), which were determined in a latter section of this paper. It is obvious that it is a difficult matter to evaluate the complexity constants from Fig. 9, as one constant can be increased if another is decreased and thus still give a good agreement between the resulting F function and the experimental points. The agreement between the experimental points and the calculated curves are not very good at high values of $c_{Ag}\,[A]^{-1}$, but this not surprising, as the [A] values which were used to draw the log F (log $[A])_{c_{Ag}}$ diagram, from which the points were interpolated, are uncertain at high values of $c_{Ag}\,[A]^{-1}$.

The Z_2, Z_3 and Z_4 functions

In the preceding section evidence for the formation of complexes of the form Ag_mA_{m+2} or $A_2(AgA)_m$ was given. In the range shown in Fig. 9, the ratio c_{Ag}/c_A was less than 6/8 in all cases due to the fact that no accurate values could be determined for [A] by the graphical integration method at higher ratios. To check if the result obtained in the preceding section is also valid for high c_{Ag}/c_A ratios, where $[Ag^+]$ but not [A] is known, Z functions where derived under assumption that the complexes formed all have the composition $A_2(AgA)_m$, except for AgA_2. The concentration of the ion AgA, the existence of which was confirmed by solubility measurements (Ref. [1]), is negligible in comparison with c_{Ag} and c_A in the whole range studied by the potentiometric measurements and is therefore omitted in the following equations as is also $[Ag^+]$.

For c_{Ag} and c_A we get

$$c_{Ag} = \beta_{1,2}\,u\,[A] + [A]^2 \sum_{m=1} m\,\beta_{m,m+2}\,u^m \tag{32}$$

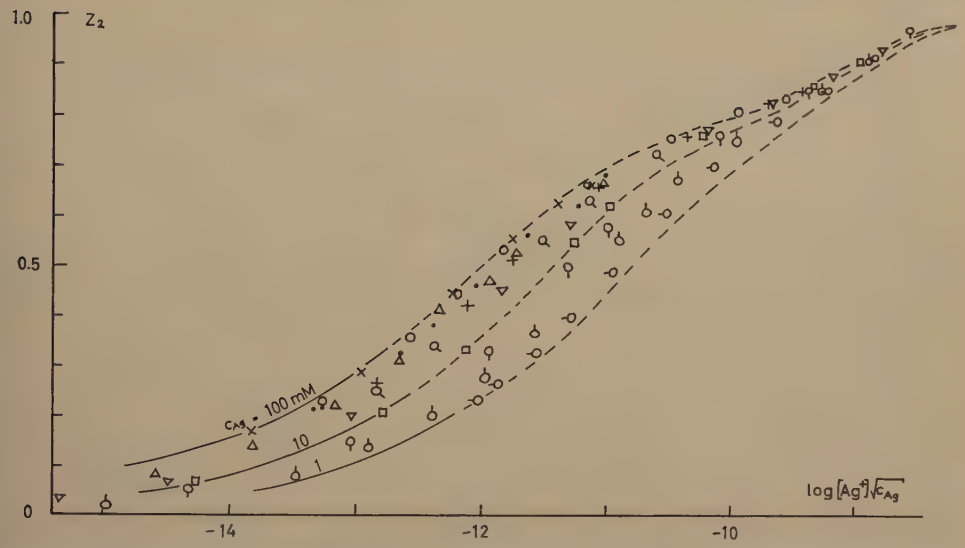

Fig. 10. The function Z_2 (defined by (34)) *vs.* $(\log [Ag^+]\sqrt{c_{Ag}})_{c_{Ag}}$. The symbols are the same as in Fig 4. Curves (broken for supersatured solutions) are calculated according to (34) for $c_{Ag} = 100$, 10, and 1 mM from the constants in Table 5. The spreading of the points from a single curve depends upon the presence of A^{2-} and AgA_2^{3-}.

$$c_A = [A] + 2\beta_{1,2} u [A] + [A]^2 \sum_{m=1} (m+2)\beta_{m,m+2} u^m \tag{33}$$

(13) (32) and (33) give

$$Z_2 = \frac{\beta_{1,2} u [A] + [A]^2 \sum_{m=1} m \beta_{m,m+2} u^m}{[A] + 2\beta_{1,2} u [A] + [A]^2 \sum_{m=1} (m+2)\beta_{m,m+2} u^m} \tag{34}$$

From (34) follows that if [A] and $[AgA_2]$ could be neglected, $[A]^2$ could be cancelled, and Z_2 would be a function of u only. u cannot be calculated for the whole range studied, but it can be expressed in known quantities in the following way. If Z_2 is held constant, u is constant (if [A] and $[AgA_2]$ are neglected), $c_{Ag} = [A]^2 \cdot$ const. and thus $u = $ const. $[Ag^+]\sqrt{c_{Ag}}$. In Fig. 10 Z_2 is plotted *vs.* $(\log [Ag^+]\sqrt{c_{Ag}})_{c_{Ag}}$ and it can be seen that the curves tend to coincide, when Z_2 rises i.e. when [A] and $[AgA_2]$ decrease. In Fig. 11 a new function

$$Z_3 = \frac{c_{Ag}}{c_A - [A]} = \frac{\beta_{1,2} u [A] + [A]^2 \Sigma\, m \beta_{m,m+2} u^m}{2\beta_{1,2} u [A] + [A]^2 \Sigma\, (m+2)\beta_{m,m+2} u^m} \tag{35}$$

is plotted against $(\log [Ag^+]\sqrt{c_{Ag}})_{c_{Ag}}$ and the points belonging to different c_{Ag} now lie on the same curve for $Z_3 > 0.8$ and nearer to each other than in Fig. 10 in the range $Z_3 < 0.8$. The spreading of the curves, which is appreciable only at low c_{Ag} values, now depends upon the presence of AgA_2. Therefore finally a function

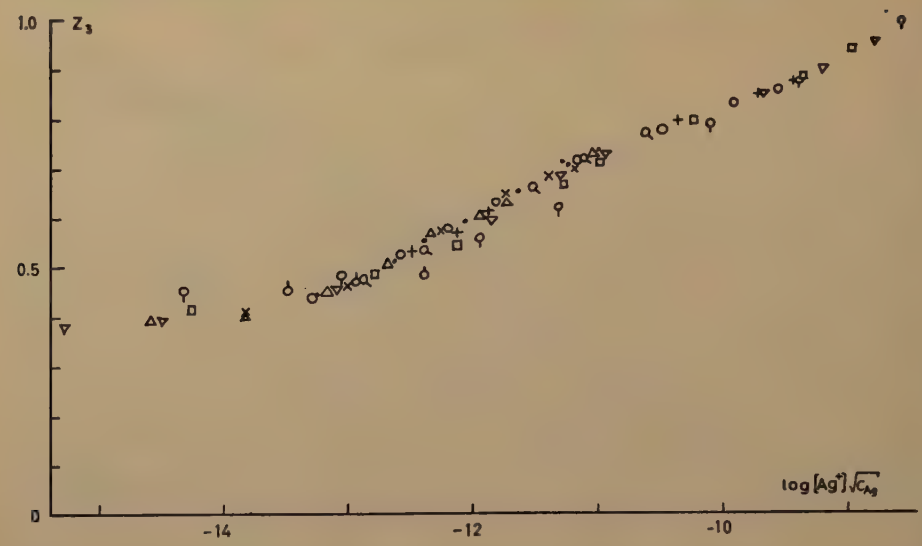

Fig. 11. The function Z_3 (defined by (35)) *vs.* $\log [Ag^+]\sqrt{c_{Ag}}$. The symbols are the same as in Fig. 4. The spreading of the points depends upon the presence of AgA_2^{3-}.

$$Z_4 = \frac{c_{Ag} - \beta_{1,2}\, u\,[A]}{c_A - [A] - 2\,\beta_{1,2}\, u\,[A]} = \frac{\sum_{m=1} m\,\beta_{m,m+2}\, u^m}{\sum_{m=1} (m+2)\,\beta_{m,m+2}\, u^m} \tag{36}$$

is plotted *versus*

$$x_p = \log [Ag^+]\sqrt{c_{Ag} - \beta_{1,2}\, u\,[A]} \tag{37}$$

in Fig. 12 ($\beta_{1,2} = 0.53 \cdot 10^{13}$). The experimental points in this diagram lie within experimental error on one and the same curve (the agreement is best at high values of Z_4 where [A] and $[AgA_2]$ only make small contributions to c_A and c_{Ag}) and this proves that the assumed series Ag_mA_{m+2} is the right one. Z_4 could also have been drawn against $[Ag^+]\sqrt{c_A - [A] - 2\,\beta_{1,2}\, u\,[A]}$, but as [A] is impaired by an error and in many ranges represents a great part of c_A it would only be of disadvantage.

The Z_4 function as expected approaches an horizontal azymptote $Z_4 = 1/3$ when c_A rises which corresponds to the mononuclear ion AgA_3. The spreading of the experimental points is great for $Z_4 \approx 1/3$. This could be foreseen as [A] and $[AgA_2]$ here constitutes a considerable part of c_A and c_{Ag}, from which they were subtracted in the calculation of Z_4 and x_p. The Z_4 curve rises when c_A decreases and formes a plateau at $Z_4 \approx 0.75$ from which one can guess that Ag_6A_8 is present. It then increases further to $Z_4 \approx 0.95$ which shows, that the polymerization continuous to high values of m. At the lowest values of c_A, [A] could not be determined according to Hedström [6]. As a first approximation Z_2 was plotted instead of Z_4 in this range ($Z > 0.88$). Then constants were looked for which roughly described the experimental curve according to reaction scheme (48) and from these preliminary constants approximate values of [A] were calculated from (53). In this range [A] is less than 1 % of c_A and $[AgA_2]$

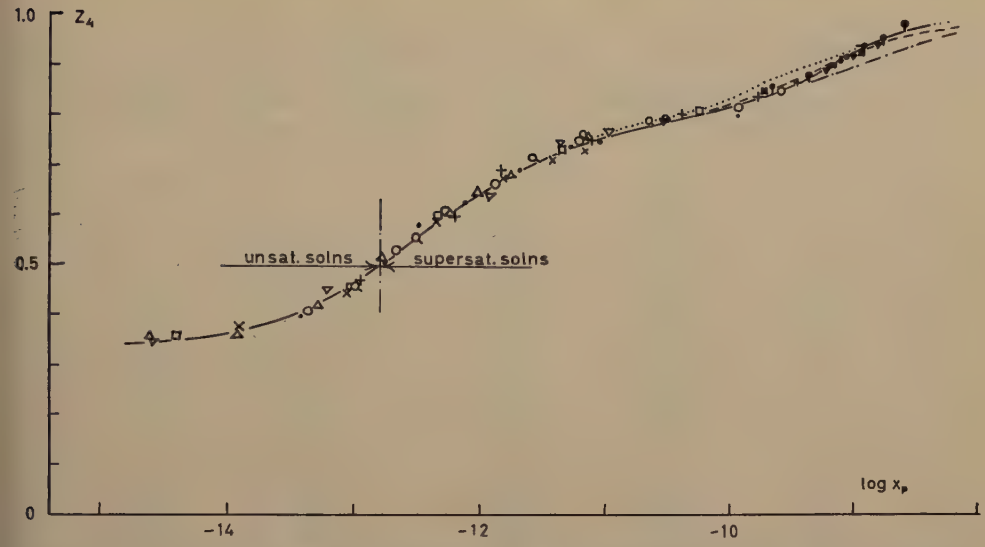

Fig. 12. The function Z_4 (defined by (36)) *vs.* log x_p (defined by (37)). Symbols as in Fig. 4. The open symbols are plotted by the aid of [A] values evaluated by the graphical integration method (12). The filled symbols are plotted by the aid of [A] values which are calculated according to (53) with preliminary $\beta_{m,n}$ constants. ——— calculated according to scheme (48) equation (59) with the constants in Table 5. – – – calculated according to scheme (48) equation (55) with the constants in Table 5. · · · · calculated according to scheme (49) equation (64). – · – · – calculated according to scheme (50) equation (69).

is less than 1 % of c_{Ag}. Therefore Z_4 and x_p could be calculated in the actual points of measurements and plotted with sufficient accuracy according to (36) and (37). Z_4 could not be calculated with any accuracy for the series with $c_{Ag}=5$, 2, and 1 mM in the range $Z_4<0.85$ as [A], representing here the main part of c_A, was afflicted with great errors. Therefore no experimental points at these silver contents could be plotted for $Z_4<0.85$. In the next section Z_4 *vs.* x_p is analyzed in a mathematical way.

Direct analysis of the Z_4-function

In order to find out which complexes are present in the series Ag_mA_{m+2} and to determine their complexity constants a direct mathematical analysis of the Z_4 *vs.* x_p function was made by analogy to the method outlined by Sillén [8]. If we introduce the symbol

$$f=\sum_{m=1}\beta_{m,m+2}\cdot u^m \tag{38}$$

Z_4 can be written

$$Z_4=\frac{uf'}{uf'+2f} \tag{39}$$

and for x_p we find

$$x_p=\log u\sqrt{uf'}=\log u+\tfrac{1}{2}\log uf' \tag{40}$$

Table 3. Corresponding values of Z_4, x_p and the quantities $\int_{-14.882}^{x_p} Z_4\, dx_p$, $\log f$, and $\log u$ evaluated by graphical integration under the curve in Figure 12.

Z_4	$-x_p$	$\int_{-14.882}^{x_p} Z_4\, dx_p$	$\log f$	$-\log u$
0.339	14.882	0	−0.906	14.434
350	14.300	0.201	−0.512	14.060
375	13.700	0.384	−0.162	13.658
400	13.493	0.502	0.057	13.583
425	13.283	0.587	0.207	13.472
450	13.097	0.676	0.366	13.392
475	12.940	0.751	0.496	13.314
500	12.805	0.817	0.608	13.260
525	12.695	0.874	0.697	13.217
550	12.560	0.945	0.816	13.163
575	12.433	1.017	0.936	13.117
600	12.300	1.094	1.063	13.071
625	12.157	1.183	1.212	13.026
650	12.000	1.282	1.380	12.977
675	11.811	1.405	1.596	12.917
700	11.640	1.524	1.798	12.873
725	11.460	1.652	2.016	12.830
750	11.195	1.848	2.367	12.770
775	10.840	2.121	2.868	12.692
800	10.460	2.421	3.417	12.620
825	10.000	2.795	4.107	12.541
850	9.686	3.062	4.573	12.500
875	9.425	3.289	4.948	12.471
900	9.195	3.489	5.253	12.449
925	9.035	3.635	5.418	12.440
950	8.855	3.806	5.584	12.436
975	8.653	3.999	5.669	12.430

From (39) we get

$$u f' = 2 f \frac{Z_4}{1 - Z_4} \tag{41}$$

and as $f' = \frac{df}{du} = \frac{df}{u\, d\ln u}$ (41) gives

$$\log u = \tfrac{1}{2} \frac{1 - Z_4}{Z_4} \log f \tag{42}$$

We now derivate (40) and eliminate u with (42). After integration we get

$$\log f = 2 \int Z_4\, dx_p + \log (1 - Z_4) + 2 C_1 \tag{43}$$

or

$$\log \frac{f_2}{f_1} = 2 \int_1^2 Z_4\, dx_p + \log \frac{1 - (Z_4)_2}{1 - (Z_4)_1} \tag{44}$$

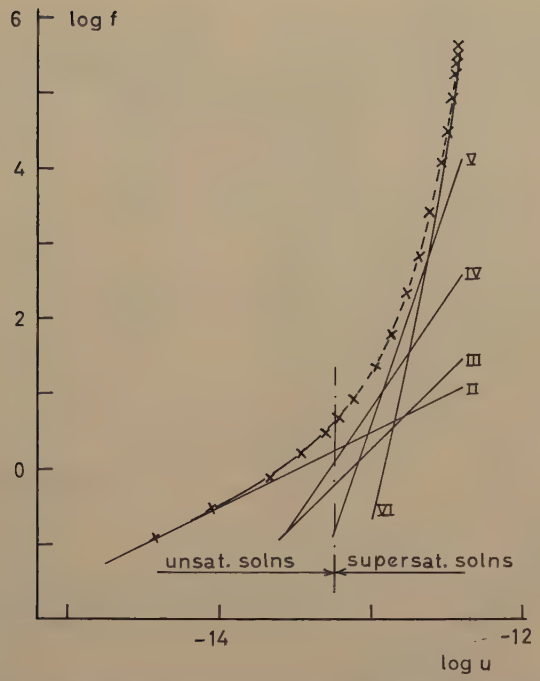

Fig. 13. log f (defined by (38)) as a function of the logarithm of the product $[Ag^+][A]$. The contribution to f by the different species can be read from the thin lines which are calculated (*cf.* (38)) with the constants in Table 5. The Roman figures represent the same complexes as in Fig. 9.

(40), (41), and (43) give

$$\log u = x_p - \int Z_4 \, d x_p - \tfrac{1}{2} \log Z_4 - \tfrac{1}{2} \log 2 - C_1 \qquad (45)$$

or

$$\log \frac{u_2}{u_1} = (x_{p2} - x_{p1}) - \int_1^2 Z_4 \, d x_p - \tfrac{1}{2} \log \frac{(Z_4)_2}{(Z_4)_1} \qquad (46)$$

A smooth curve is drawn through the experimental points and the graphical integration is started at the point $Z_4 = 0.339$; $x_p = -14.882$ where $u_1 = 3.68 \cdot 10^{-15}$ and $f_1 = 0.124$. The value for u_1 has been calculated according to the following equation derived from (32) and (37):

$$x_p = \log \left(\Sigma \, m \, \beta_{m, m+2} \, u^{m+2} \right)^{\frac{1}{2}} \qquad (47)$$

and f_1 has been calculated from (38). The already evaluated $\beta_{1,3}$ constant and preliminary values for $\beta_{m,m+2}$ $(m > 1)$ have been used for these calculations. The $\beta_{m,m+2}$ values have been obtained in a trial and error experiment, carried out in order to find the constants which could describe the Z_4 function. At the starting point the mononuclear term makes up about 97 % of f_1 and the error arising from uncertain values of the polynuclear constants is therefore small.

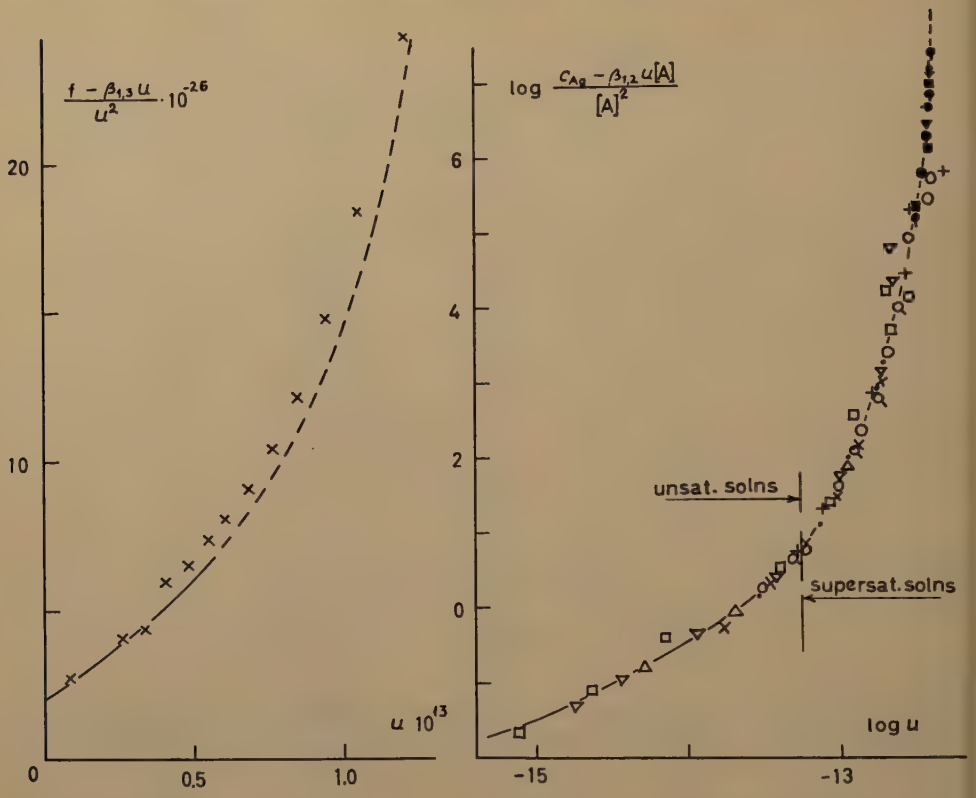

Fig. 14 (left). Evaluation of the $\beta_{2,4}$ constant from the function f (defined by (38)). The points have been plotted with $\beta_{1,3}=3.3\cdot 10^{13}$. The curve (broken for supersaturated solutions) is calculated according to (38) with the constants in Table 5.

Fig. 15 (right). $\log (c_{Ag}-5.3\cdot 10^{12}\cdot u\,[A])\,[A]^{-2}$ *vs.* the logarithm of the product $[Ag^+][A]$. Symbols as in Fig. 4. The curve is calculated according to (57) with the constants in Table 5. The open symbols are plotted with [A] data obtained according to (12). Filled symbols plotted with [A] data calculated from (57) and (58) with preliminary $\beta_{m,m+2}$ constants.

Now $\int_{-14.882}^{x_p} Z_4\,dx_p$ are determined by graphical integration in Fig. 12 and corresponding values of u and f are then calculated according to (44) and (46). In Table 3 Z_4, x_p, $\int_{-14.882}^{x_p} Z_4\,dx_p$, $\log f$, and $\log u$ are given and in Fig. 13 $\log f$ *vs.* $\log u$ is plotted. The curve approaches at decreasing u-values the slope 1, which means that AgA_3 dominates. At increasing values of u, however, the slope increases and the experimental curve seems to approach a vertical azymtote, which means that the complex formation does not stop at a certain value of m, but probably continues to very high numbers of m.

The f-function is now analysed in a linear manner. In Fig. 14 $(f-\beta_{1,3}u)\,u^{-2}$ is plotted *vs.* u with $\beta_{1,3}=3.3\cdot 10^{13}$ (determined in a preceding section). From

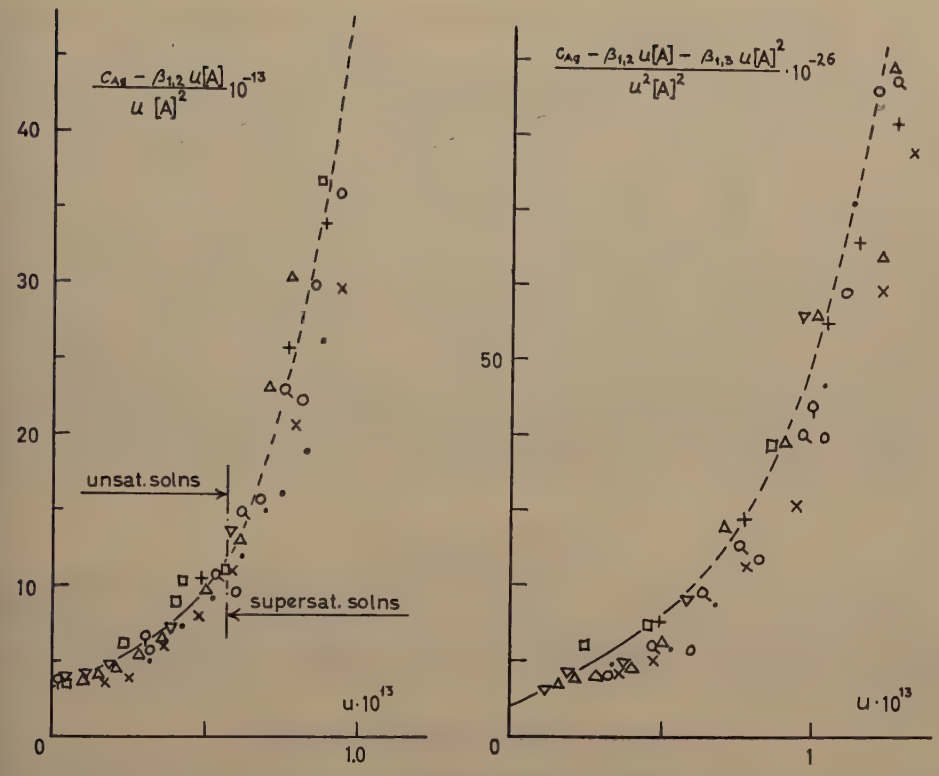

Fig. 16 (left). Evaluation of $\beta_{1,3}$. The experimental points have been plotted with $\beta_{1,2} = 5.3 \cdot 10^{12}$. The curve (broken for supersaturated solutions) is calculated according to (57) with the values from Table 5.

Fig. 17 (right). Evaluation of $\beta_{2,4}$. The experimental points have been plotted with $\beta_{1,2} = 5.3 \cdot 10^{12}$ and $\beta_{1,3} = 3.3 \cdot 10^{13}$. The curve is calculated according to (57) with the constants in Table 5.

the ordinate when $u = 0$ one obtains $\beta_{2,4} = 2 \cdot 10^{26}$. This constant is relatively small and shows that Ag_2A_4 contributes at most 15 % of the silver content. This appears to be too small an amount for determining the existence and strength of this complex. An attempt was therefore made to extrapolate the curve in Fig. 14 to origo by taking $\beta_{1,3} = 3.5 \cdot 10^{13}$. This caused the curve to attain a concave shape, however, thereby losing its physical meaning.

Then $(f - \beta_{1,3}\,u - \beta_{2,4}\,u^2)\,u^{-3}$ *vs.* u was drawn (not shown here) and an extrapolation of the curve gave the origo ordinate $\beta_{3,5} = 7 \cdot 10^{39}$. Finally

$$(f - \beta_{1,3}\,u - \beta_{2,4}\,u^2 - \beta_{3,5}\,u^3)\,u^{-6}$$

was drawn *vs.* u and the extrapolated curve gave $\beta_{6,8} = 4 \cdot 10^{78}$. The constants $\beta_{4,6}$ and $\beta_{5,7}$ were taken to be zero because they seemed to have only small values, if they are not actually zero. It was not possible to evaluate the constants $\beta_{m,m+2}$ with $m > 6$ in a similar way as the errors became too great.

The constants $\beta_{m,m+2}$ can also be obtained more directly from the experimental points by examination of $(c_{Ag} - \beta_{1,2}\, u\,[A])\,[A]^{-2}$ which according to (32) is a function of u only. In Fig. 15 $\log (c_{Ag} - \beta_{1,2}\, u\,[A])\,[A]^{-2}$ is plotted against $\log u$ and it can be seen that the experimental points fall on a single curve within the experimental error. The spreading of the open symbols is great at high u values. This is not surprising, as accurate [A] values could not be obtained in this range with the integration method. Fig. 15 also shows that u approaches a vertical azymtote $u = 4 \cdot 10^{-13}$, a value only seven times higher than $u = k_{1,-1} = 0.57 \cdot 10^{-13}$, which holds for solutions (with $[Na^+] = 4$ M) saturated with $NaAgA \cdot H_2O$ (Ref. [1]). This explains why the measurements could be extended so far into the supersaturated field (*vide* Fig. 20). A linear extrapolation is made in Fig. 16 where $(c_{Ag} - \beta_{1,2}\, u\,[A])\,[A]^{-2}\, u^{-1}$ is drawn *versus* u. The origo ordinate gives $\beta_{1,3} = 3.3 \cdot 10^{13}$ which is in agreement with the value found in an earlier section. In Fig. 17 $(c_{Ag} - \beta_{1,2}\, u\,[A] - \beta_{1,3} \cdot u\,[A]^2)\,(u\,[A])^{-2}$ is plotted *vs.* u and the experimental curve can be extrapolated to the origo ordinate $2\,\beta_{2,4} = 4 \cdot 10^{26}$ which is in accordance with the value found by extrapolating the f-function.

The range $m > 6$

It is not possible to obtain reliable information concerning the constants in $f = \Sigma\, \beta_{m,m+2}\, u^m$ for $m > 6$ by analysing this function in the way mentioned above. The further polymerization ($m > 6$) must therefore be described on a more or less loose assumption. Here the following three mechanisms were tried out ($m \geqslant 6$).

$$Ag_{3z}A_{3z+2} + Ag_3A_5 \rightleftarrows Ag_{3(z+1)}A_{3z+5} + 2\,A \tag{48}$$

$$Ag_mA_{m+2} + AgA_2 \rightleftarrows Ag_{m+1}A_{m+3} + A \tag{49}$$

$$Ag_{6z}A_{6z+2} + Ag_6A_8 \rightleftarrows Ag_{6z+6}A_{6z+8} + 2\,A \tag{50}$$

Mechanism (48) was tried because Ag_4A_6 and Ag_5A_7 could be neglected and Ag_2A_4 was a weak complex. The complexes are accordingly assumed to be built up stepwise by addition of three silver atoms and three thiosulphate groups in every step. The equilibrium constant for $z = 2$ is called h_0 and the following constants are called h. Thus

$$[Ag_9A_{11}] = h_0\,[Ag_6A_8]\,[Ag_3A_5]\,[A]^{-2}$$

and

$$[Ag_{3(z+1)}A_{3z+5}] = h\,[Ag_{3z}A_{3z+2}]\,[Ag_3A_5]\,[A]^{-2} = h_0\,h^{-1}\,[Ag_6A_8]\,(h\,[Ag_3A_5]\,[A]^{-2})^{z-1} \qquad (z \geqslant 3) \tag{51}$$

If

$$h\,[Ag_3A_5]\,[A]^{-2} = h\,\beta_{3,5} \cdot u^3 = y_1 \tag{52}$$

we get (y_1 is always < 1)

$$c_{Ag} = \sum_{m=1}^{6} m[Ag_mA_n] + h_0 \cdot h^{-1}\,[Ag_6A_8] \sum_{z=2} 3\,(z+1)\,y_1^{z-1} =$$

$$= \sum_{m=1}^{3} m\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[6 + h_0 \cdot h^{-1}\,y_1\,(9 - 6\,y_1)\,(1 - y_1)^{-2}] \tag{53}$$

($\Sigma\, z\, y_1^{z-1}$ is the derivate of a geometrical series!) and similarly

$$c_A = [A] + \sum_{m=1}^{3} n\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[8 + h_0 \cdot h^{-1}\,y_1\,(11 - 8\,y_1)\,(1-y_1)^{-2} \quad (54)$$

Z_4 is now obtained from (36), (53), and (54)

$$Z_4 = \frac{\sum_{m=1}^{3} m\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[6 + h_0\,h^{-1}\,y_1\,(9 - 6\,y_1)\,(1-y_1)^{-2}]}{\sum_{m=1}^{3} (m+2)\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[8 + h_0\,h^{-1}\,y_1\,(11 - 8\,y_1)\,(1-y_1)^{-2}]} \quad (55)$$

When Z_4 was drawn against x_p it was found that the best agreement with the experimental points was obtained with $h_0 = 0.0074$ and $h = 0.0022$. The curve calculated with these constants and with other constants from Table 5 is shown in Fig. 12. As the agreement is not complete for high Z_4 values it was assumed that the value of the equilibrium constant in (48) was not h but

$$[1 - (z+1)^{-1}]\,h \quad (56)$$

for $z \geqslant 3$. In this case we get

$$c_{Ag} = \sum_{m=1}^{3} m\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[6 + 9\,h_0\,h^{-1}\,y_1\,(1-y_1)^{-1}] \quad (57)$$

$$c_A = [A] + \sum_{m=1}^{3} n\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[8 + 9\,h_0\,h^{-1}\,y_1\,(1-y_1)^{-1} + 3\,h_0\,h^{-1}\,y_1^{-2}\,\{4\,(1-y_1) - (1-y_1)^2 - 2\,\ln\,(1-y_1) - 3\}] \quad (58)$$

$$Z_4 = \frac{\sum_{1}^{3} m\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[6 + 9\,h_0\,h^{-1}\,y_1\,(1-y_1)^{-1}]}{\sum_{1}^{3} (m+2)\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[8 + 9\,h_0\,h^{-1}\,y_1\,(1-y_1)^{-1} + 3\,h_0\,h^{-1}\,y_1^{-2} \cdot \{4\,(1-y_1) - (1-y_1)^2 - 2\,\ln\,(1-y_1) - 3\}]} \quad (59)$$

Z_4 calculated according to (59) with the constants in Table 5 is in better agreement with the experimental points in Fig. 12, than is the curve calculated according to (55) with the same constants.

It was also tried to describe the Z_4 function with the aid of *scheme* (49). In this case it was assumed that

$$[Ag_7A_8] = b_0\,[Ag_6A_8]\,[AgA_2]\,[A]^{-1} \quad \text{and}$$

$$[Ag_{m+1}A_{m+3}] = b\,[Ag_mA_{m+2}]\,[AgA_2]\,[A]^{-1} = b_0\,b^{-1}\,[Ag_6A_8]\,(b\,[AgA_2]\,[A]^{-1})^{m-5} \quad (60)$$

for $m \geqslant 7$. If

$$b\,[AgA_2]\,[A]^{-1} = b\,\beta_{1,2}\,u = y_2 \quad (61)$$

we get (y_2 always < 1)

$$c_{Ag} = \sum_{m=1}^{6} m\,[Ag_m A_n] + b_0\, b^{-1}\,[Ag_6 A_8] \sum_{m=6} (m+1)\, y_2^{m-5} =$$

$$= \sum_{m=1}^{3} m\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[6 + b_0\,b^{-1}\,y_2\,(7-6\,y_2)\,(1-y_2)^{-2}] \quad (62)$$

$$c_A = [A] + \sum_{m=1}^{3} n\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[8 + b_0\,b^{-1}\,y_2\,(9-8\,y_2)\,(1-y_2)^{-2}] \quad (63)$$

and

$$Z_4 = \frac{\sum_{m=1}^{3} m\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[6 + b_0\,b^{-1}\,y_2\,(7-6\,y_2)\,(1-y_2)^{-2}]}{\sum_{m=1}^{3} (m+2)\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[8 + b_0\,b^{-1}\,y_2\,(9-8\,y_2)\,(1-y_2)^{-2}]} \quad (64)$$

Best results were obtained with $b_0 = b = 0.55$ (see Fig. 12), but the agreement was not so good as with scheme (48).

Finally and attempt was made to describe the curve above $Z_4 \approx 0.75$ with the aid of scheme (50). In this case we get

$$[Ag_{12} A_{14}] = g_0\,[Ag_6 A_8]^2\,[A]^{-2} \quad \text{and}$$

$$[Ag_{6(z+1)} A_{6z+8}] = g\,[Ag_{6z} A_{6z+2}]\,[Ag_6 A_8]\,[A]^{-2} = g_0\,g^{-1}\,[Ag_6 A_8]\,(g\,[Ag_6 A_8]\,[A]^{-2})^z \quad (z \geq 2) \quad (65)$$

If

$$g\,\beta_{6,8}\,u^6 = y_3 \quad (66)$$

we get (y_3 always < 1)

$$c_{Ag} = \sum_{m=1}^{6} m\,[Ag_m A_n] + g_0\,g^{-1}\,[Ag_6 A_8] \sum_{z=1} 6\,(z+1)\,y_3^z =$$

$$= \sum_{m=1}^{3} m\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[6 + g_0\,g^{-1}\,2\,y_3\,(6-3\,y_3)\,(1-y_3)^{-2}] \quad (67)$$

$$c_A = [A] + \sum_{m=1}^{3} n\,\beta_{m,n}\,u^m\,[A]^{n-m} + \beta_{6,8}\,u^6\,[A]^2\,[8 + g_0\,g^{-1}\,2\,y_3\,(7-4\,y_3)\,(1-y_3)^{-2}] \quad (68)$$

$$Z_4 = \frac{\sum_{m=1}^{3} m\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[6 + g_0\,g^{-1}\,2\,y_3\,(6-3\,y_3\,(1-y_3)^{-2}]}{\sum_{m=1}^{3} (m+2)\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[8 + g_0\,g^{-1}\,2\,y_3\,(7-4\,y_3)\,(1-y_3)^{-2}]} \quad (69)$$

Best result was obtained with $g_0 = 3 \cdot 10^{-4}$ and $g = 3 \cdot 10^{-5}$ (see Fig. 12) but the calculated curve did not coincide with the experimental points for $Z_4 > 0.8$.

The silver distribution

The amount of the silver present as $Ag_m A_n$ can be calculated from

$$\alpha_{m,n} = \frac{m\,\beta_{m,n}\,[Ag^+]^m\,[A]^n}{c_{Ag}} \quad (70)$$

The distribution of the silver in different complexes is shown in Fig. 18 as a function of log [A] and c_A for $c_{Ag} = 100$, 10, 1, and 0 mM.

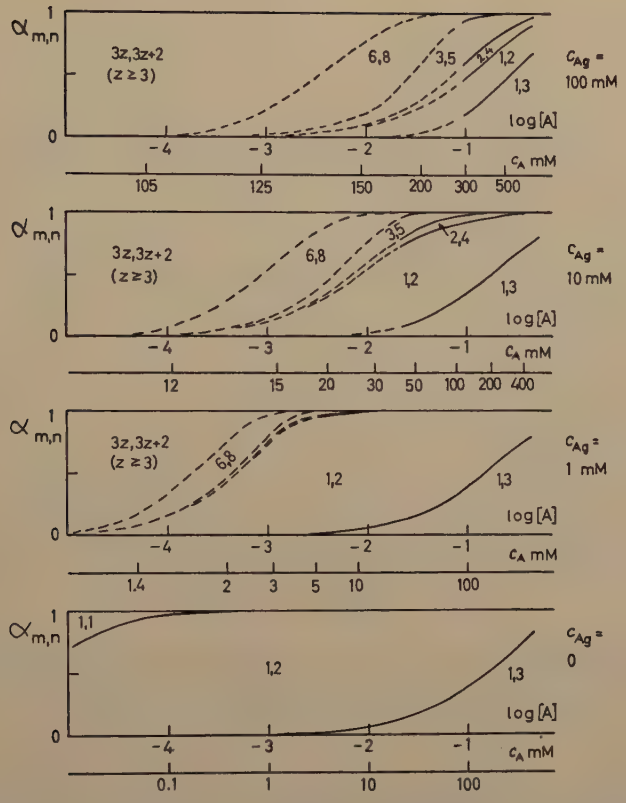

Fig. 18. The distribution of the silver on different complexes. The curves (broken for supersaturated solutions) are calculated according to (70) with the constants from Table 5. If a vertical line is drawn at a certain value of log [A] or c_A (which is an analytically known quantity) the length of this line, falling in a section marked for example 1,2 gives that part of the total silver which is present as AgA_2.

The distribution of the silver belonging to the series Ag_mA_{m+2} in different values of m at a given value of u can be calculated from

$$\alpha_s = \frac{m\,[Ag_mA_{m+2}]}{c_{Ag} - [AgA_2]} = \frac{m\,\beta_{m,m+2}\,u^m}{\Sigma\, m\,\beta_{m,m+2}\,u^m} \tag{71}$$

For the scheme (48), which best describes the measurements, we get (*cf.* (57))

$$\alpha_s = \frac{m\,\beta_{m,m+2}\,u^m}{\sum_1^3 m\,\beta_{m,m+2}\,u^m + \beta_{6,8}\,u^6\,[6 + 9\,h_0\,h^{-1}\,y_1\,(1-y_1)^{-1}]} \tag{72}$$

For $m \geqslant 9$ we get from (56) and (51) ($3z = m$)

$$\beta_{3z,3z+2} = 3\,z^{-1}\,h_0\,h^{-1}\,\beta_{6,8}\,(h\,\beta_{3,5})^{z-2} \tag{73}$$

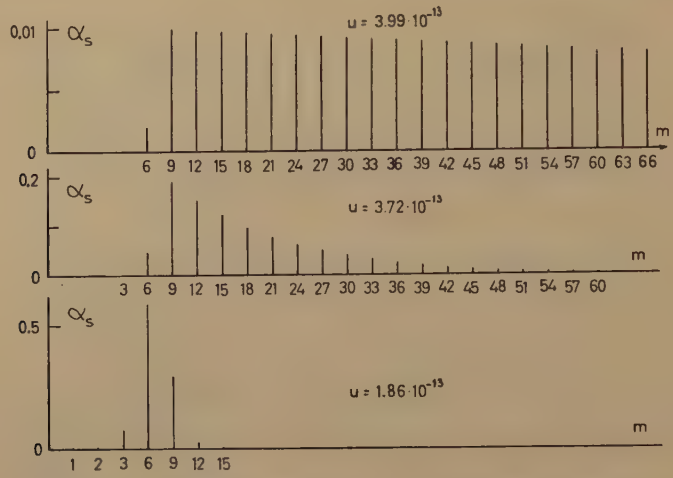

Fig. 19. The distribution on different values of m of that part of the silver, which is present as Ag_mA_{m+2}, is shown. The amount of the total silver content which is present as Ag_mA_{m+2} ($=c_{Ag}-[AgA_2]$) can be evaluated from Fig. 18 by the aid of Table 4, which gives corresponding values of c_{Ag}, u, and [A]. The conditions at $u = 0.57 \cdot 10^{-13}$ (saturated solutions) are shown in Ref. [1].

Table 4. Corresponding values of c_{Ag}, u, and [A] (in mM) calculated according to (57) with the constants in Table 5.

c_{Ag} mM \ u M²	$1.86 \cdot 10^{-13}$	$3.72 \cdot 10^{-13}$	$3.99 \cdot 10^{-13}$
100	7.27	0.267	0.0446
10	2.08	0.0846	0.0141
1	0.524	0.0262	0.00446

Fig. 19 shows three distribution diagrams drawn according to (72). When u rises, *i.e.* when supersaturation increases, the number of silver atoms in each ion increases. At $u = 3.99 \cdot 10^{-13}$, which is reached in the series with $c_{Ag} = 5$ mM, we get very large ions. So for example when $m = 210$, $\alpha_{m,s}$ is still as high as 0.005, which means that about half as many silver atoms are bound in complex ions with $m = 210$ as in ions with $m = 9$.

The log $[Ag^+]$ (log $c_A)_{c_{Ag}}$ curves

The agreement between the calculated curves in the Figs. 4 and 9 and the experimental points is not too good, especially at low c_A values. This discrepancy is not surprising, however, as the [A] values are uncertain at high c_{Ag}/c_A ratios, especially when the c_{Ag} values are low. To check the evaluated constants against a measured quantity, $[Ag^+]_{c_{Ag}}$ was calculated as a function of c_A ac-

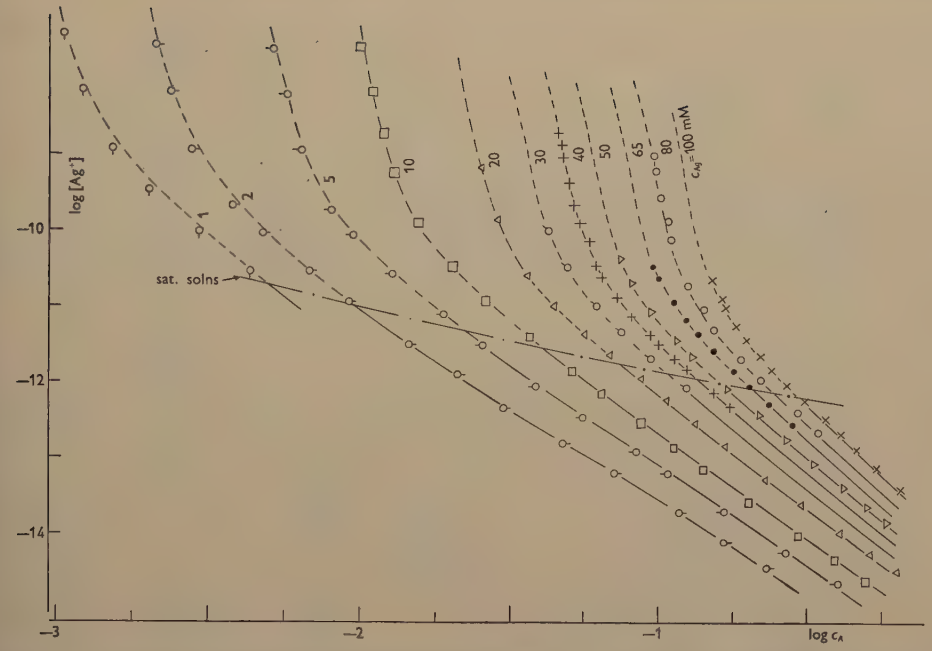

Fig. 20. The free silver ion concentration as a function of the total thiosulphate concentration at constant total silver content shown in logarithmic scale. The curves, which are broken for supersaturated solutions, are calculated according to (57) and (58) from the constants in Table 5.

cording to (57) and (58). These functions together with every third experimental point are shown in a logarithmic scale in Fig. 20. The agreements is satisfactory except for c_{Ag} 1, 2, and 5 mM where the calculated curves differ from the experimental points in a medium c_A range. It is not possible to avoid this discrepancy by assuming a new value for E_{m_0} as a change in E_{m_0} only causes a parallel shift of the curve, but does not change its form. Therefore it was tried to eliminate this discrepancy by introducing new complexes, which according to (31) and Fig. 9 must be of the form $A(AgA)_m$ or $(AgA)_m$ as these complexes should only dominate at low silver and low thiosulphate concentrations. As the last term in (57) approaches c_{Ag} when u approaches $u_{max.}$ and the contribution to c_{Ag} by the complexes Ag_mA_m are given by $m\beta_{m,m}u^m$, which is independent of [A], the constants $\beta_{m,m}$ must be zero.

With the aid of the series $A(AgA)_m$ on the other hand it is possible to eliminate the discrepancy in Fig. 20 over the medium range of thiosulphate concentration. For example the following assumption was found to be useful.

$$Ag_6A_7 + Ag_3A_5 \rightleftarrows Ag_9A_{10} + 2A\,;\ [Ag_9A_{10}] = [Ag_6A_7]\,d\,[Ag_3A_5]\,[A]^{-2} = [Ag_6A_7]\,d\cdot j \quad (74)$$

$$Ag_{3z}A_{3z+1} + Ag_3A_5 \rightleftarrows Ag_{3z+3}Ag_{3z+4} + 2A\,;\ [Ag_{3z+3}A_{3z+4}] = [Ag_{3z}A_{3z+1}]\,d\cdot j = $$
$$= [Ag_6A_7]\,(dj)^{z-1} \qquad (z \geqslant 2) \quad (75)$$

If
$$dj = y_4 \quad (76)$$

Table 5. The constants $\beta_{m,n}$ used to describe the potentiometric measurements. Ionic strength 4 M ($NaClO_4$). Temperature 25°C.

Reaction	Equilibrium constant	$\beta_{m,n}$ defined by (7)[1]	$k_{m,x} = \beta_{m,n}\, k_{1,-1}^{m}$ [2]
$AgA^- + A^{3-} \rightleftarrows AgA_2^{3-}$	$2.3 \cdot 10^5$	$\beta_{1,2} = (5.3 \pm 0.5)\, 10^{12}$	$k_{1,1} = 0.30$
$AgA_2^{3-} + A^{2-} \rightleftarrows AgA_3^{5-}$	6.2	$\beta_{1,3} = (3.3 \pm 0.5)\, 10^{13}$	$k_{1,2} = 1.9$
$2\,AgA_3^{5-} \rightleftarrows Ag_2A_4^{6-} + 2\,A^{2-}$	0.2	$\beta_{2,4} = 2 \cdot 10^{26}$	$k_{2,2} = 0.7$
$Ag_2A_4^{6-} + AgA_3^{5-} \rightleftarrows Ag_3A_5^{7-} + 2\,A^{2-}$	1	$\beta_{3,5} = 7 \cdot 10^{39}$	$k_{3,2} = 1.3$
$2\,Ag_3A_5^{7-} \rightleftarrows Ag_6A_8^{10-} + 2\,A^{2-}$	0.09	$\beta_{6,8} = 4.2 \cdot 10^{78}$	$k_{6,2} = 0.14$
$Ag_6A_8^{10-} + Ag_3A_5^{7-} \rightleftarrows Ag_9A_{11}^{13-} + 2\,A^{2-}$	$h_0 = 0.0074$	$\beta_{9,11} = h_0 \cdot \beta_{6,8}\, \beta_{3,5} = 2 \cdot 10^{116}$	$k_{9,2} = 0.0013$
$Ag_{3z-3}A_{3z-1}^{(3z-2)-} + Ag_3A_5^{7-} \rightleftarrows$	$\left(1-\frac{1}{z}\right) h =$	$\beta_{3z,3z+2} = \frac{h_0}{h}\, 3\, z^{-1}\, \beta_{6,8}\, (h\, \beta_{3,5})^{z-2} =$	$k_{3z,2} = 1.4\, z^{-1}\, 0.0028^{z-2}$
$Ag_{3z}A_{3z+2}^{(3z+4)-} + 2\,A^{2-}$	$= \left(1-\frac{1}{z}\right) 0.0022$	$= 4.2 \cdot 10^{79}\, z^{-1}\, (1.5 \cdot 10^{37})^{z-2}$	$(z \geqslant 3)$
$(z \geqslant 4)$	$(z \geqslant 4)$	$(z \geqslant 3)$	$K_2 = \Sigma\, m\, k_{m,x} = 8.0$

[1] *Cf.* discussion concerning the influence of NaA^- complexes.

[2] The solubility measurements in Ref. [1] could be described by the constants $K_0 = 1.3 \cdot 10^{-6}$ (corresponding to $\beta_{1,1} = 2.3 \cdot 10^7$), $K_1 = 0.30$, $K_2 = 8$, and $K_5 = 17\,000$.

we get

$$c_{Ag} = [\beta_{1,2}\, u + \beta_{6,7}\, u^6 (6 - 3y_4)(1 - y_4)^{-2}][A] + [A]^2 \sum_{m=1} m\, \beta_{m,m+2}\, u^m \qquad (77)$$

$$c_A = [1 + 2\beta_{1,2}\, u + \beta_{6,7}\, u^6 (7 - 4y_4)(1 - y_4)^{-2}][A] + [A]^2 \sum_{m=1} (m+2)\, \beta_{m,m+2}\, u^m \qquad (78)$$

Curves calculated according to (77) and (78) with the constants from Table 5 and from $\beta_{6,7} = 4 \cdot 10^{75}$ and $d = 0.0015$, could describe the experimental points in Fig. 20 for the whole range of measurements except for the lowest thiosulphate concentrations. The contribution to K_1 (see (6) in Ref. [1]) by the complexes $A(AgA)_m$ ($m \geqslant 6$) is given by

$$\Delta K_1 = \sum_{m=6} m\, \beta_{m,m+1} \cdot k_{1,-1}^m = \beta_{6,7}\, k_{1,-1}^6 [6 + 3y_5(3 - 2y_5)(1 - y_5)^{-2}] = 0.001 \qquad (79)$$

where $y_5 = d\, \beta_{3,5}\, k_{1,-1}^3 = 0.002$. From the solubility measurements, $K_1 = 0.30$ was evaluated (Ref. [1]) and it has been shown in the present paper that $k_{1,1} = \beta_{1,2}\, k_{1,-1} = 0.30$. The small value of ΔK_1 does consequently not exclude the possibility that a series $A(AgA)_m$ really exists, *but it must in spite of this be excluded*, as it does not fit the experimental points at the lowest values of c_A. It thus seems probable that the discrepancy in Fig. 20 is due to activity changes or it may be that the electrodes do not attain the correct potentials at the lowest c_{Ag} values.

Discussion

The exchange of perchlorate ions by thiosulphate ions may have an unfavorable influence upon the constancy of the activity coefficients. Measurements by Leden [9] give an idea of the magnitude of this influence. He studied the complex formation in the silver sulphate system, which resembles the silver thiosulphate system in that the anions have the same charges, and could describe his experimental values up to $c_{SO_4} = 0.6$ M with $\beta_{1,1} = 1.7$ and $\beta_{1,2} = 1.9$ when sodium perchlorate had been added to the ionic strength 3 M and with $\beta_{1,1} = 1.7$ and $\beta_{1,2} = 1.0$ when the sodium ion concentration was kept constant at 3 M. In the present work more sodium perchlorate was added ($I = 4$ M) and only solutions with $c_{S_2O_3} \leqslant 0.3$ M were taken into consideration. Moreover the most interesting part of the complex formation, the polymerization reactions, took place at the lowest thiosulphate concentrations and the series Ag_mA_{m+2} chosen to describe the measurements is therefore probably the right one.

From the potentiometric measurements in this paper the complexity constants for the ions AgA_2^{3-}, AgA_3^{5-}, $Ag_2A_4^{6}$, and $Ag_{3z}A_{3z+2}^{(3z+4)-}$ ($z \geqslant 1$) have been evaluated. They are collected in Table 5 where they have also been recalculated to the corresponding $k_{m,x}$ constants. That the series $Ag_mA_{m+2}^{(m+4)-}$, chosen to describe the measurements is the right one, is indicated by the results obtained from the F functions in Fig. 9 and from the fact that the experimental points in Fig. 12 all lie on the same curve, independent of the total silver concentration to which they belong. The next step, to determine which complexes are present in this series and to evaluate their complexity constants is, however, a harder problem. In the silver thiocyanate system (Ref. [4]) the formation of polynuclear complexes could be explained with only two constants. It is at once obvious that more than two constants must be used here, as the Z_4 func-

tion (in Fig. 12) formes a plateau at $Z_4 \approx 0.75$. This plateau indicates that the ratio $m:n$ is $\approx 0\,75$ for the ions $Ag_mA_n^{(2n-m)-}$ in the solution, and one may therefore guess that the ion $Ag_6A_8^{10-}$, which exhibits this ratio is especially stable. One should remember, however, that it is difficult to decide from these kinds of measurements whether $Ag_6A_8^{10-}$ or equal parts of $Ag_5A_7^{9-}$ and $Ag_7A_9^{11-}$ are present in the solution. The range $1/3 < Z_4 < 0.75$ was described in this paper with the aid of the complexes AgA_3^{5-}, $Ag_2A_4^{6-}$, $Ag_3A_5^{7-}$, and $Ag_6A_8^{10-}$ with constants obtained by linear extrapolation of suitable functions as described in an earlier section. In the range $0.75 < Z_4 < 1$ on the other hand, three different reaction schemes (48), (49), and (50) were tried. The first of them, involving addition of one $Ag_3A_5^{7-}$ group and a simultaneous splitting off of two thiosulphate ions in every condensation step, seems to best explain the experimental results. This may indicate that a reaction according to (48), which was chosen because Ag_2A_4, Ag_4A_6, and Ag_5A_7 seemed to be weak complexes while Ag_3A_5 and Ag_6A_8 are strong (see Fig. 18), really takes place. It should be noted, however, that also other reactions could possibly explain the measurements if suitable values of the complexity constants were chosen. A comparison between the crystal structures of different thiosulphates with structure models of the ions formed in the solution would be of interest. Unfortunately the structures of $Na_3AgA_2 \cdot 2\,H_2O$, $Na_5Ag_3A_4 \cdot 2\,H_2O$, and $NaAgA \cdot H_2O$ (or corresponding silver thiosulphates containing other alkali metals) are not known. It is a well-known fact that the structure of a complex ion in a crystal does not always correspond to the ions of the solution in equilibrium with it. For instance solutions in contact with $NaAgA \cdot H_2O$ contain AgA^-, AgA_2^{3-}, AgA_3^{5-}, or polynuclear complexes, depending upon the thiosulphate concentrations of the solutions (*cf.* Fig. 4 in Ref. [1]). On the other hand it is interesting to note that the above-mentioned crystals, which together with Ag_2A and $NaAg_3A_2 \cdot H_2O$ are the only existing ones in the sodium silver thiosulphate system [1], all have stoichiometric compositions corresponding to ions formed in the solutions. Thus $Na_3AgA_2 \cdot 2\,H_2O$ corresponds to AgA_2^{3-} and $Ag_2A_4^{6-}$, $Na_5Ag_3A_4 \cdot 2\,H_2O$ to $Ag_6A_8^{10-}$, and $NaAgA \cdot H_2O$ to AgA^- and $Ag_mA_{m+2}^{(m+4)-}$ $(m \to \infty)$. It would not be surprising if $NaAgA \cdot H_2O$, which precipitates from the supersaturated solutions contains infinite ions $Ag_mA_{m+2}^{(m+4)-}$ $(m \to \infty)$ as such ions, with very high values of m (*cf.* Fig. 19) are present in the supersaturated solutions.

In the previous discussion the possible presence of the ion NaA^- have been disregarded. The formation constant for this ion is as high as $\beta_{NaA^-} = 4\text{–}5$ at the ionic strength of zero. This value has been evaluated from conductivity and solubility measurements by Denney and Monk [10] and from spectrophotometric measurements by Gimblett and Monk [11]. If this constant is of the same order of magnitude in the ionic medium used here, it would mean that the ratio $[NaA]:[A]$ would be $\beta_{NaA}[Na^+] \approx 15$ as $[Na^+]$ lies in the range $3.4 - 4\,M$ when c_A decreases from $0.3\,M$ to zero (*cf.* (1)). To examine how a sodium thiosulphate complex would influence the $\beta_{m,n}$ constants, equation (12) is derived under assumption that the species NaA^- is present. Formula (2) in Hedströms paper [6] then becomes ($[A_t]$ = true free thiosulphate concentration)

$$c_A = [A_t] + [NaA] + \Sigma\, n\, \beta_{m,n(t)}\, [Ag^+]^m\, [A_t]^n$$

or

$$c_A = [A_t]\,(1 + \beta_{NaA}\,[Na^+]) + \Sigma\, n\, \beta_{m,n(t)}\, [Ag^+]^m\, [A_t]^n$$

but from the latter, (9) and (11 a) are derived unchanged if $[Na^+]$ is kept constant. When (11 a) is integrated it must be observed that we have the boundary condition:

$$c_{Ag}=0; \quad \log \frac{[A_t]}{c_A} = -\log (1+\beta_{NaA}[Na^+])$$

and we get

$$\log \frac{[A_t]}{c_A} = -\frac{F}{RT}\left[\int_0^{c_{Ag}} \left(\frac{\partial E}{\partial c_A}\right)_{c_{Ag}} d c_{Ag}\right]_{c_{Ag}} - \log (1+\beta_{NaA}[Na^+])$$

This means that the quantity [A] obtained by the graphical integration in this paper is the true free thiosulphate concentration, $[A_t]$, plus the concentration of NaA^- if NaA^- is present, thus $[A]=[A_t](1+\beta_{NaA}[Na^+])$. The expression for the evaluated complexity constants consequently becomes $\beta_{m,n}=[Ag_mA_n][Ag^+]^{-m}\{[A_t](1+\beta_{NaA}[Na^+])\}^{-n}$. The true constants for the silver thiosulphate complexes then are $\beta_{m,n(t)}=\beta_{m,n}(1+\beta_{NaA}[Na^+])^n$. For the solubility measurements (Ref. [1]) $[A]=[A_t](1+\beta_{NaA}[Na^+])$ holds, and the true equilibrium constants are accordingly $k_{m,x(t)}=k_{m,x}(1+\beta_{NaA}[Na^+])^x$.

It is possible that β_{NaA^-} is much smaller at the ionic strength used here, than at the ionic strength of zero. This can be guessed from the fact that β for the ion TlA^-, which has the same charge as NaA^-, has the value 100 at low ionic strength (Novakovskiĭ and Ryazantseva [12]) while its value at the ionic strength of 4 M has been evaluated to 7 (Nilsson [13]).

As the aim of the present paper is to find out what kind of complexes are formed, rather than to determine their absolute complexity constants, the value of β_{NaA^-} is of less interest in this connection. The discrepancy between the solubility measurements at the upper part of the solubility curve and the potentiometric measurements can only partly be explained by the presence of the NaA^- complex and it must therefore be ascribed to activity changes. This is not surprising, since the ionic mediums are different in the two methods of measurements.

Symbols used in this paper

M	g moles/liter.
[A]	$=[S_2O_3^{2-}]$, concentration of free thiosulphate ions.
c_A	total concentration of thiosulphate.
c_{Ag}	total concentration of silver.
E_m	emf of cell (2).
E	emf of cell (5).
$\beta_{m,n}$	complexity constant for Ag_mA_n, defined by (7).
$\bar{n}$	ligand number, number of thiosulphate groups bound to each silver atom (10).
F	$=c_{Ag}[Ag^+]^{-1}$ (28).
$F_{m,n}$	defined by (30).
X	$=(c_{Ag}[Ag^+]^{-1})_{c_{Ag}=0}$ (11).
K_x	$=\Sigma\, m\, k_{m,x}$, equilibrium constants (see (4) and (6) in Ref. [1]).
R	defined by (14).
u	$=[Ag^+][A]$ (21).
f	$=\Sigma\, \beta_{m,m+2}\, u^m$ (38).
x_p	defined by (37).
Z_0	number of silver atoms bound to each thiosulphate group, defined by (13).
Z_1	defined by (25).
Z_2	defined by (34).
Z_3	defined by (35).
Z_4	defined by (36).

c equilibrium constant, defined by (26).
h_0 ,, ,, ,, ,, (51).
h ,, ,, ,, ,, (51).
b_0 ,, ,, ,, ,, (60).
b ,, ,, ,, ,, (60).
g_0 ,, ,, ,, ,, (65).
g ,, ,, ,, ,, (65).
d ,, ,, ,, ,, (74).
y_1 $= h\,\beta_{3,5}\,u^3$ defined by (52).
y_2 $= b\,\beta_{1,2}\,u$,, ,, (61).
y_3 $= g\,\beta_{6,8}\,u^6$,, ,, (66).
y_4 $= d\,\beta_{3,5}\,u^3$,, ,, (74) and (76).
y_5 $= d\,\beta_{3,5}\,k^3_{1,-1}$ (defined by (79).
$\alpha_{m,n}$ the amount of the silver present as Ag_mA_n (70).
α_s defined by (71).

SUMMARY

The complex formation between silver and thiosulphate ions at 25°C and the ionic strength 4 M (with $NaClO_4$ as additional salt) was studied by potentiometric titration at which $[Ag^+]$ was measured with silver electrodes as a function of the total thiosulphate concentration. The total silver concentration was kept constant in each series. The free thiosulphate concentration was evaluated according to Hedström [6]. The measurements could be explained by assuming the complexes $Ag(S_2O_3)_2^{3-}$, $Ag(S_2O_3)_3^{5-}$, $Ag_2(S_2O_3)_4^{6-}$, and $Ag_{3z}(S_2O_3)_{3z+2}^{(3z+4)-}$ $(z \geqslant 1)$. The complexity constants are given in Table 5. The distribution of the silver is shown in Figs. 18 and 19.

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The complex formation between thallium(I) ions and thiosulphate ions

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With 6 figures in the text

The complex formation between thallium(I) ions and thiosulphate ions has been studied earlier by Euler [1], who made potentiometric measurements and suggested the presence of $Tl(S_2O_3)_2^{3-}$. Brintzinger and Eckardt [2] report $Tl_2(S_2O_3)_2^{2-}$ by dialysis measurements. The formation of this ion could not be confirmed by the present investigations however. Novakovskiĭ and Ryazantseva [3] measured the solubility of $Tl_2S_2O_3$ in sodium thiosulphate solutions and evaluated $\beta_{1,1} = 100$ for $TlS_2O_3^-$ at low ionic strength and 25°C.

The present investigations deal with: A) potentiometric measurements of $[Tl^+]$ as a function of $c_{S_2O_3}$ with c_{Tl} constant in each series, B) solubility determinations on $Tl_2S_2O_3$ (s) in thallium perchlorate and sodium thiosulphate solutions, C) solubility determinations on $NaTlS_2O_3 \cdot z\,H_2O$ (s) in sodium thiosulphate solutions. All measurements were carried out at 25°C and 4 M ionic strength.

Apparatus and chemicals

The *4 M $NaClO_4$ solution*, the *thallium perchlorate* and the *thallium amalgam* were prepared as described in Ref. [4].

4/3 M $Na_2S_2O_3$ was made according to Ref. [5].

$Tl_2S_2O_3$(s) was precipitated from a hot diluted thallium nitrate solution by the addition of an equivalent amount of sodium thiosulphate solution. The precipitate was filtered off after 12 h, washed with hot water and dried. The thallium content was determined by precipitating it as chromate. The thiosulphate content was determined by titration with iodine solution.

	mmol/g	
	found	calc.
Tl	3.843	3.840
S_2O_3	1.937	1.920

$NaTlS_2O_3 \cdot z\,H_2O$ (s). To 1 litre 0.5 M $Na_2S_2O_3$ 0.1 mol $TlNO_3$ at 35°C was added, forming long, white, flexible needles on cooling. Such needles have previously been described in literature, but opinions concerning their composition differ. Thus Werther [6] proposed the formula $3\,Na_2S_2O_3 \cdot 2\,Tl_2S_2O_3 \cdot 10\,H_2O$, Jochum [7] $3\,Na_2S_2O_3 \cdot 2\,Tl_2S_2O_3 \cdot 8\,H_2O$, and Vortman and Padborg [8], finally, $2\,Na_2S_2O_3 \cdot Tl_2S_2O_3 \cdot 8\,H_2O$. The crystals obtained under the present investigations were pressed between filter papers and then dried to constant weight over P_4O_{10}. By ti-

tration with KJO_3 in 3–6 M HCl with JCl as indicator (see Swift and Garner [9]) Tl(I) was oxidized to Tl(III) and $S_2O_3^{2-}$ to SO_4^{2-}. The latter reaction was confirmed by titrating known thiosulphate solutions under similar conditions. The sulphate ions were precipitated and weighed as $BaSO_4$. The thallium content was also determined by precipitation as Tl_2CrO_4 and the thiosulphate content by titration with iodine. The results differed at the most by 0.5 % from the ones obtained by the first method. After the crystals were dried at 110°C it could be shown by Guinier photographs that their structures had changed. At 180°C the crystals darkened and their structures changed again.

	Found		Calc. for $NaTlS_2O_3 \cdot 2\,H_2O$
	mmole/g	mg/g	mmole/g
Tl	2.670	545.7	2.663
S_2O_3	2.735	306.7	2.663
Na	2.80	64.4	2.663
H_2O (110°)	3.33	59.9	5.326
		976.7	

If the rest, 23.3 mg, is assumed to be water which did not leave the structure at 110°C, the total water content becomes 4.62 m mole/g and we obtain $Na : Tl : S_2O_3 : H_2O = 1.05 : 1 : 1.025 : 1.72$. From this follows that the formula probably is $NaTlS_2O_3 \cdot 2\,H_2O$. Novakovskiĭ and Ryazantseva [3] also report that they obtained $NaTlS_2O_3$ as a gel by the interaction of a concentrated $Na_2S_2O_3$ solution and a saturated $Tl_2S_2O_3$ solution, and that the compound was dehydrated at 105–110°C and decomposed at 160–180°C.

The *reference electrodes* were the same as described in Ref. [4].

The emf measurements were made with a Jensen compensator (type 10 A) and a Norma galvanometer.

A. Potentiometric measurements

The measurements were made in the form of potentiometric titrations at which $[Tl^+]$ was measured with a thallium amalgam electrode as a function of the total thiosulphate concentration, c_A. The total thallium content, c_{Tl}, was kept constant within each series. Measurements were carried out on the following cell (A stands for S_2O_3):

$$-\,\mathrm{Tl,\ Hg}\ \Big|\ \begin{matrix} c_{Tl}\ \mathrm{M\ TlClO_4} \\ c_A\ \mathrm{M\ Na_2A} \\ (4 - c_{Tl} - 3\,c_A)\ \mathrm{M\ NaClO_4} \end{matrix}\ \Big|\ \begin{matrix} 4\ \mathrm{M\ NaClO_4} \\ \text{or} \\ 4/3\ \mathrm{M\ Na_2A} \end{matrix}\ \Big|\ \begin{matrix} 4\ \mathrm{M} \\ \mathrm{NaClO_4} \end{matrix}\ \Big|\ \mathrm{Hg_2SO_4,\ Hg} + \qquad (1)$$

The emf of this cell was

$$E_m = E_0 - 59.16 \log [Tl^+] = 1097.2 - 59.16 \log [Tl^+] \qquad (2)$$

E_0 was determined from measurements in cell (1) when $c_A = 0$. It was thereby found that the emf of the cell followed Nernst's law for $c_{Tl} > 1$ mM (*cf.* Table 2 in Ref. [4]).

The following calculations were based on the equation

$$E = E_m - E_{m_0} = 59.16 \log \frac{c_{Tl}}{[Tl^+]} \qquad (3)$$

where E_{m_0} is the emf of cell (1) when $c_A = 0$. Five titration series were made with $c_{Tl} = 2.44$, 4.88, 9.77, 19.5, and 39.1 mM respectively. In the first of these

Table 1. Corresponding values of c_A, E, and [A]. $\beta_{1,1} = 7.2\ M^{-1}$, $\beta_{1,2} = 5.2\ M^{-2}$, $\beta_{1,3} = 1.5\ M^{-3}$, and $\beta_{2,4} = 350\ M^{-5}$.

$c_{Tl} = 2.44$ mM			$c_{Tl} = 4.88$ mM			$c_{Tl} = 9.77$ mM			$c_{Tl} = 19.5$ mM			$c_{Tl} = 39.1$ mM		
c_A mM	E mV	[A] mM	c_A mM	E mV	[A] mM	c_A mM	E mV	[A] mM	c_A mM	E mV	[A] mM	c_A mM	E mV	[A] mM
13.2	2.5	13.0	13.2	2.4	12.8	13.2	2.2	12.4	474	44.4	455	1100	73.9	1040
26.1	4.5	25.7	63.4	10.1	61.9	51.1	7.9	48.3	498	45.8	479	1165	76.3	1105
51.3	8.5	50.6	94.1	13.9	92.1	110	15.4	105	553	49.0	532	1237	79.0	1177
75.4	11.7	74.5	136	18.6	134	193	24.1	186	622	53.0	600	1286	80.6	1226
104	15.3	103	208	25.6	204	299	32.9	291	751	59.0	726	1320	81.8	1260
121	17.0	120	249	29.1	245	387	39.1	377	829	62.6	802			
163	21.4	162	327	35.4	322	477	44.8	467	905	65.8	877			
198	24.9	196	381	39.1	376	557	49.3	546	1076	72.7	1045			
222	27.0	220	499	46.2	494	665	54.7	653	1137	75.0	1106			
308	33.8	306	602	51.8	596	831	62.2	817	1244	78.8	1211			
381	39.0	379	784	60.3	777	950	67.2	936	1327	81.7	1290			
487	45.4	484	888	64.5	881	1025	70.1	1010						
613	52.3	610	951	67.2	944	1108	73.3	1092						
820	62.1	817	1150	74.8	1140	1210	77.1	1195						
1000	69.6	996	1280	79.6	1273	1330	81.4	1313						

series a solution c_{Tl} M $TlClO_4$, $(4 - c_{Tl})/3$ M Na_2A was added to a solution c_{Tl} M $TlClO_4$, $(4 - c_{Tl})$ M $NaClO_4$, while in the two last series the titrations were carried out in the reverse order. In the series with c_{Tl} 4.88 and 9.77 mM titrations were made in both directions, and the emfs differed by at most 0.2 mV between the two methods. Nitrogen was bubbled through the solutions during the measurements. In Table 1, E (calculated according to (3)) is given as a function of c_A. The figures are the average from at least two measurements.

If the complexity constants are defined as

$$\beta_{m,n} = \frac{[Tl_m A_n]}{[Tl^+]^m [A]^n} \tag{4}$$

we get for the total thallium content

$$c_{Tl} = [Tl^+] + \sum_{m=1} \sum_{n=1} m \beta_{m,n} [Tl^+]^m [A]^n \tag{5}$$

and for the total thiosulphate content

$$c_A = [A] + \sum_{m=1} \sum_{n=1} n \beta_{m,n} [Tl^+]^m [A]^n \tag{6}$$

The ligand number defined as

$$\bar{n} = \frac{c_A - [A]}{c_{Tl}} \tag{7}$$

is here calculated according to

$$\bar{n} = \frac{1}{59.16} \left(\frac{\partial E}{\partial \log [A]} \right)_{c_{Tl}} \approx \frac{1}{59.16} \left(\frac{\Delta E}{\Delta \log c_A} \right)_{c_{Tl}} \tag{8}$$

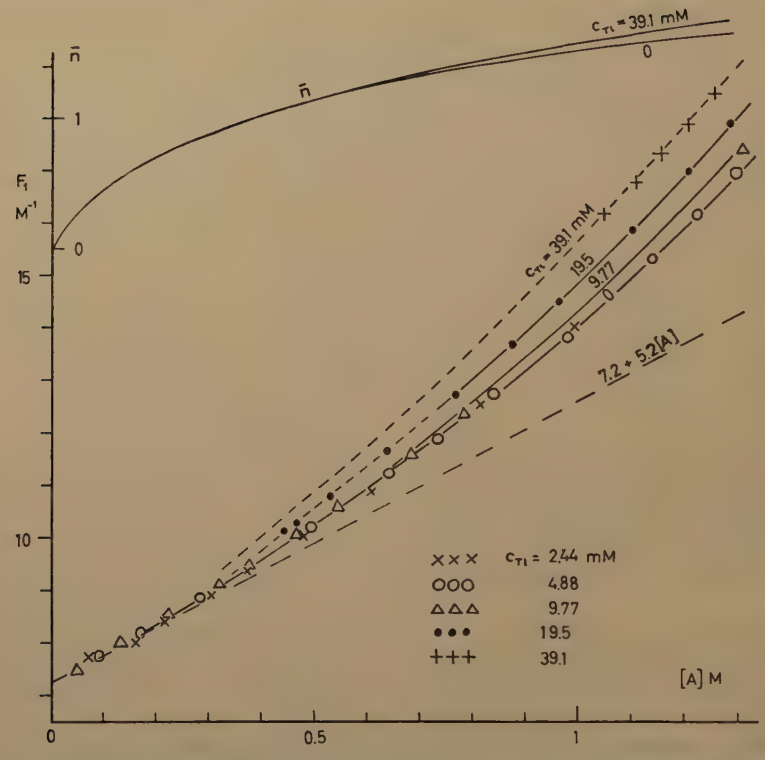

Fig. 1. F_1 (defined by equation (9)) and the ligand number $\bar{n}$ as functions of the free thiosulphate concentration. The equation of the straight line is $F_1 = \beta_{1,1} + \beta_{1,2}[A]$. The drawn F_1-curves (broken lines for supersaturated solutions) are calculated according to (5) and (9) with the potentiometric constants in Table 4.

(*cf.* Bjerrum [10]). The equation (8) is valid, if only mononuclear complexes are present. $\bar{n}$ was plotted against c_A and straight lines with slopes c_{Tl}^{-1} were drawn (*cf.* (7)), which intersected the curve in the points $(\bar{n}; c_A)$. The intercepts between the c_A axis and the straight lines then gave the [A] values corresponding to the c_A values in the points of intersection with the $\bar{n}$-curve. For $c_{Tl} > 5$ mM it was found, that polynuclear complexes were also formed (*cf.* Fig. 1) and consequently the free thiosulphate concentrations as calculated in the above way are not quite correct. Here preliminary complexity constants were evaluated by the aid of the free thiosulphate concentrations obtained in the way described, then $\bar{n}$ was calculated according to (7), (5), and (6) with the preliminary constants and drawn *vs.* [A], and finally straight lines with slopes $-c_{Tl}^{-1}$ were drawn, which intersected the [A] axis in the points c_A. The corresponding values of [A] were then read off from the points of intersection with the $\bar{n}$-curve.

For evaluation of the complexity constants a function

$$F_1 = \frac{F-1}{[A]} = \beta_{1,1} + \beta_{1,2}[A] + \beta_{1,3}[A]^2 + \cdots + \sum_{m=2} \sum_{n=0} m\beta_{m,n}[Tl^+]^{m-1}[A]^{n-1} \qquad (9)$$

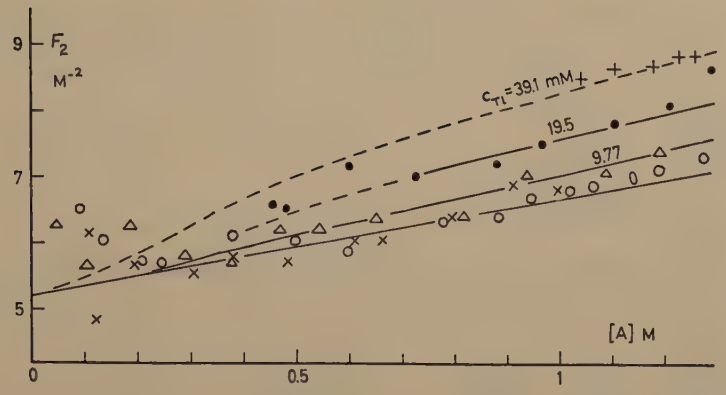

Fig. 2. The function F_2 (defined by equation (10)) *versus* the free thiosulphate concentration. The symbols are the same as in Fig. 1. The drawn curves (broken lines for supersaturated solutions) have been calculated according to (5) and (10) with the potentiometric constants in Table 4.

is formed (*cf.* Leden [11]). From (9) follows that F_1 is independent of c_{Tl} if only mononuclear complexes are present. In Fig. 1 this appears to be the case for $c_{Tl} < 10$ mM, while for the two highest thallium contents the experimental points lie above the curve which is calculated for $c_{Tl} = 0$. Extrapolation of F_1 to $[A] = 0$ gives $\beta_{1,1} = 7.2$. In Fig. 2

$$F_2 = \frac{F_1 - \beta_{1,1}}{[A]} = \beta_{1,2} + \beta_{1,3}[A] + \cdots + \sum_{m=2} \sum_{n=0} m \beta_{m,n} [Tl^+]^{m-1} [A]^{n-2} \qquad (10)$$

is plotted, giving for $c_{Tl} < 10$ mM a straight line. The intersection with the ordinate axis gives $\beta_{1,2} = 5.2$ and the slope of the line gives $\beta_{1,3} = 1.5$.

The experimental points belonging to c_{Tl} 19.5 and 39.1 mM lie above the straight line $\beta_{1,2} + \beta_{1,3}[A]$ in Fig. 2 and this shows that here also polynuclear complexes are present (provided the activity factors are kept constant). An effort was made to find out what kind of polynuclear complexes exists, by forming a function

$$F_2 - (\beta_{1,2} + \beta_{1,3}[A]) = \sum_{m=2} \sum_{n=0} m \beta_{m,n} [Tl^+]^{m-1} [A]^{n-2} \qquad (11)$$

This function $F_2 - (\beta_{1,2} + \beta_{1,3}[A])$ was plotted *vs.* $[Tl^+][A]^2$ and the experimental points did approximately fall on a straight line with the slope 700 through origo. The slope of this line is then according to (11) equal to $2\beta_{2,4} = 700$. The constants which were evaluated in this section, are given in Table 4.

The distribution of silver in the different complexes is shown in Fig. 3 at thallium concentrations of 0 and 39.1 mM. The above distribution has been calculated according to (5) and (12).

$$\alpha_{m,n} = \frac{m[Tl_m A_n]}{c_{Tl}} = \frac{m \beta_{m,n} [Tl^+]^m [A]^n}{[Tl^+] + \sum_m \sum_n m \beta_{m,n} [Tl^+]^m [A]^n} \qquad (12)$$

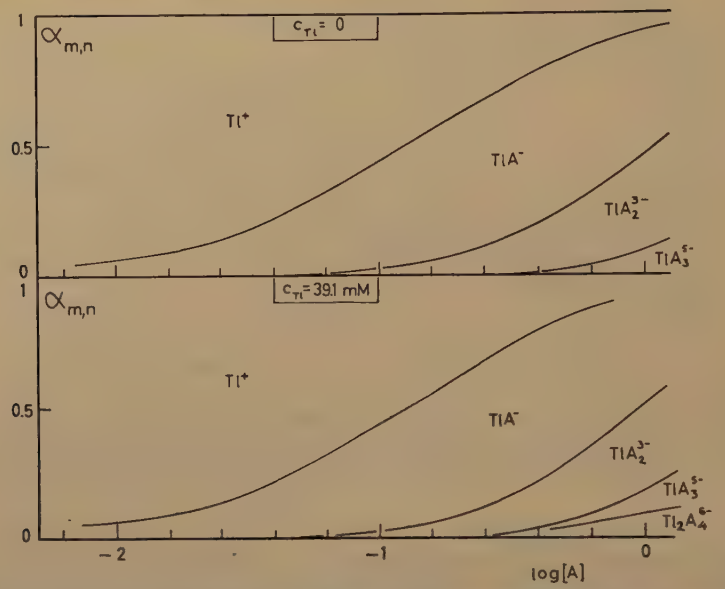

Fig. 3. The distribution of the thallium on different complexes at infinite dilution and at 39.1 mM as a function of the logarithm of the free thiosulphate concentration. The curves have been calculated according to (5) and (12) with the potentiometric constants in Table 4.

B. Solubility measurements on Tl_2A (s)

In order to check the constants evaluated from the potentiometric measurements in the preceding section of this paper, the solubility of Tl_2A (s) was determined in sodium thiosulphate solutions with $c_{Na_2A} \leqslant 20$ mM (the upper limit was set by the fact that Tl_2A is transformed to $NaTlA \cdot z\,H_2O$ at [A] = 28 mM), and in thallium perchlorate solutions with $c_{TlClO_4} < 100$ mM (the upper limit was set by the low solubility product of $TlClO_4$). The thallium thiosulphate, Tl_2A (s), was transferred into tubes containing solutions with compositions c_{Na_2A} M Na_2A, $(4 - 3\,c_{Na_2A})$ M $NaClO_4$ and c_{TlClO_4} M $TlClO_4$, $(4 - c_{TlClO_4})$ M $NaClO_4$, respectively. Nitrogen was bubbled through the solutions to avoid oxidation by air, and the tubes were then shaken for a period of three to six days. The saturated solutions were filtered through colloid filters in a thermostatically controlled Thiessen apparatus. The thallium in the filtrates was precipitated and weighed as Tl_2CrO_4. In some of the filtered solutions the thiosulphate content was determined by iodine titration. The agreement (see Table 2) between the thiosulphate content determined in this way and the value calculated according to

$$c_A = c_{Na_2A} + l/2 \qquad (13)$$

($l/2$ is the solubility of Tl_2A) shows, that no decomposition has taken place and that the presence of thiosulphate ions does not disturb the determination of the thallium by the method used.

In the saturated solutions $[Tl^+]$ was determined by emf measurements in cell (1), the saturated solutions used in the left half cell.

Table 2. The solubility of Tl_2A (s) in thallium perchlorate and sodium thiosulphate solutions at 25°C. Ionic strength 4 M. $h_{1,1} = 2.1 \cdot 10^{-4}\ M^2$.

c_{Na_2A} mM	c_{TlClO_4} mM	c_{Tl} mM	c_A calc. mM	c_A exp. mM	$[Tl^+]$ mM	[A] mM	$h_{0,2}$ M^3 10^5
		(17)	(13)		(2)	(25)	(20)
0	99.8	109.3	4.8	4.6	107.2	2.6	(3.0)
0	89.8	100.4	5.3		98.3	3.2	(3.1)
0	74.9	88.1	6.6		86.0	4.5	(3.3)
0	59.9	76.5	8.3		73.9	5.7	(3.1)
0	44.9	66.5	10.8	10.7	63.4	7.7	(3.1)
0	29.9	58.0	14.0		54.0	10.0	2.92
0	20.0	53.1	16.6	16.7	48.7	12.2	2.89
0	10.0	48.9	19.5		44.4	15.0	2.95
0	4.7	47.2	21.3	21.4	42.3	16.4	2.94
0	0	45.9	22.9		40.6	17.6	2.90
0	0	46.0	23.0	22.6	40.4	17.4	2.85
2	0	44.7	24.4		39.3	19.0	2.93
4	0	43.6	25.8	25.7	37.8	20.0	2.85
6	0	42.5	27.3		36.6	21.4	2.86
8	0	41.4	28.7		35.5	22.8	2.88
10	0	40.6	30.3	30.0	34.6	24.3	2.90
12	0	39.8	31.9		33.6	25.7	2.90
20[1]	0	37.2	38.6	38.7	30.4	31.8	2.93
							Mean 2.90

[1] Supersaturated solution (*cf.* Fig. 5).

X-ray photographs (Guinier camera) showed that the solid phase, which remained in the tubes after saturation, consisted of Tl_2A in all cases.

If the formation of the complexes is expressed by the equation

$$n\,Tl_2A\,(s) \rightleftharpoons Tl_m\,A_n^{(2n-m)-} + (2n-m)\,Tl^+ \tag{14}$$

the system is defined by the constants

$$h_{m,y} = [Tl_m\,A_n^{y-}]\,[Tl^+]^y \tag{15}$$

where

$$y = 2n - m \tag{16}$$

is the negative charge of the complexes. For the total thallium content we get

$$c_{Tl} = l + c_{TlClO_4} = [Tl^+] + \sum_m \sum_y m\,h_{m,y}\,[Tl^+]^{-y} = [Tl^+] + \sum_y H_y\,[Tl^+]^{-y} \tag{17}$$

where for odd values of y

$$H_y = h_{1,y} + 3\,h_{3,y} + 5\,h_{5,y} + \cdots \tag{18}$$

and for even values of y

$$H_y = 2\,h_{2,y} + 4\,h_{4,y} + 6\,h_{6,y} + \cdots \tag{19}$$

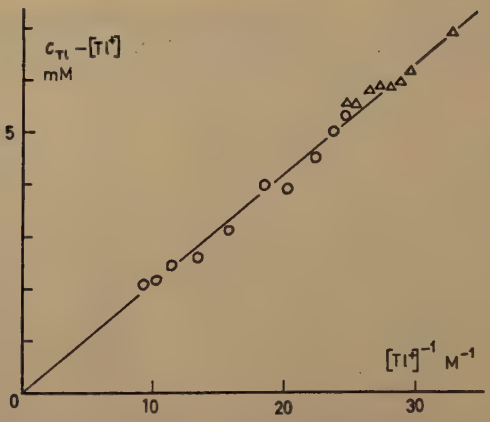

Fig. 4. The amount of thallium bound in complexes as a function of the reciprocal of the free thallium ion concentration in solutions saturated with Tl_2A (s). Symbols as in Fig. 5.

From (17), (18), and (19) it follows that from corresponding values of c_{Tl} and $[Tl^+]$ only the sums H_y can be determined but not the individual constants $h_{m,y}$.

In Fig. 4 $c_{Tl} - [Tl^+]$ is plotted against $[Tl^+]^{-1}$ and the experimental points lie with good approximation on a straight line with the slope $H_1 = 2.10 \cdot 10^{-4}$ through origo. This means that in the solutions studied in this section, only complexes which are represented by the constants $h_{1,1}$, $h_{3,1}$, $h_{5,1}$, *etc.* can be present. In solutions saturated with respect to Tl_2A the solubility product is

$$h_{0,2} = [Tl^+]^2 [A] \tag{20}$$

and from (4), (15), (16), and (20) we get

$$\beta_{m,n} = h_{m,y} (h_{0,2})^{-n} \tag{21}$$

For the comparison of $\beta_{m,n}$ with $h_{m,y}$ we therefore have to know the value of $h_{0,2}$, and for calculating this quantity we must also know [A]. Therefore [A] was in the first approximation calculated according to (5) with the constants evaluated from the potentiometric measurements, and in this way $h_{0,2} = 2.9 \cdot 10^{-5}$ was obtained. The value $\beta_{1,1} = 7.2$ was then recalculated according to (21) giving $h_{1,1} = 2.1 \cdot 10^{-4}$ which agrees with the value of H_1 evaluated in Fig. 4. From this follows that $h_{3,1}$, $h_{5,1}$, $h_{7,1}$, *etc.* can be neglected in comparison with $h_{1,1}$. Now [A] and consequently also $h_{0,2}$ can be calculated in a more straightforward way. For the total thiosulphate content we have the equation

$$c_A = [A] + \sum_y G_y [Tl^+]^{-y} \tag{22}$$

where for odd values of y

$$G_y = \frac{y+1}{2} h_{1,y} + \frac{y+3}{2} h_{3,y} + \cdots \tag{23}$$

and for even values of y

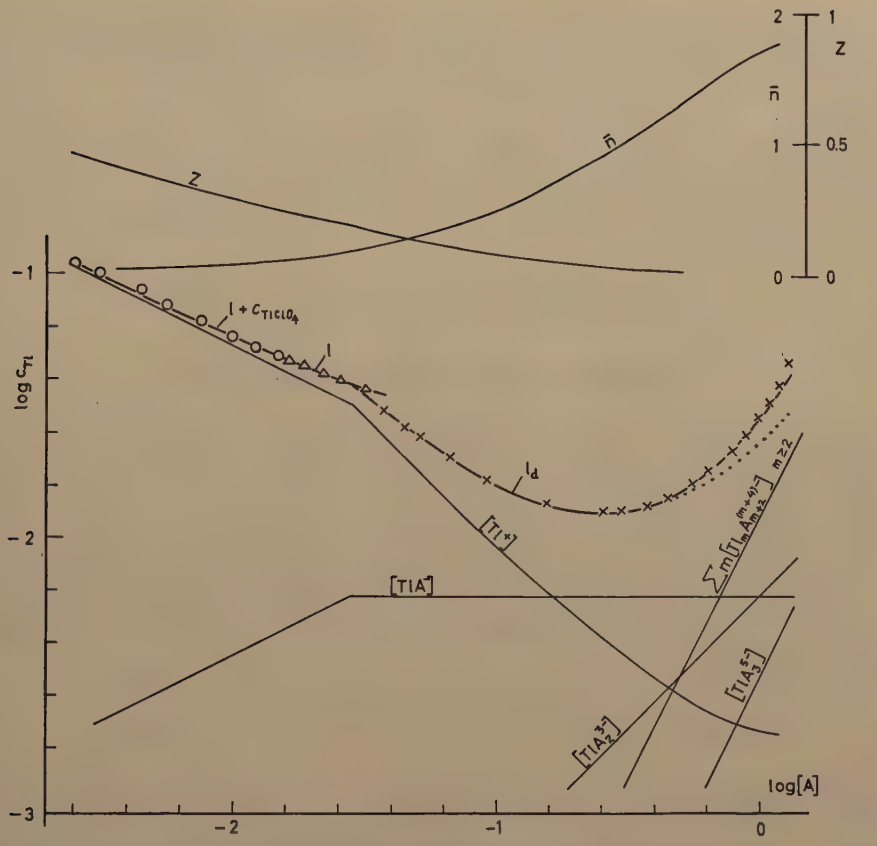

Fig. 5. The logarithm of the total thallium concentration (see (17), (29)), Z (defined by equation (35)), and the ligand number $\bar{n}$ (equation (26), (33)) *vs.* the logarithm of the free thiosulphate concentration. ○ ○ ○ $TlClO_4$-solutions saturated with Tl_2A (s). △ △ △ Na_2A-solutions saturated with Tl_2A (s). × × × Na_2A-solutions saturated with $NaTlA \cdot z\,H_2O$ (s). The concentration of a complex Tl_mA_n at a certain value of log [A] can be read off in logarithmic scale from the line marked with the symbol of this complex ion. These lines have the slopes $n-m$ in the range where the solutions are saturated with $NaTlA \cdot z\,H_2O$ (s) and the slopes $n-m/2$ in the range where the solid phase is Tl_2A (s). The dotted curve is calculated with the constants evaluated from the potentiometric measurements.

$$G_y = \frac{y+2}{2} h_{2,y} + \frac{y+4}{2} h_{4,y} + \cdots \tag{24}$$

Since the only existing complex in the concentration range studied is TlA^-, we get $G_1 = H_1 = h_{1,1}$. From (13), (17), and (22) we obtain

$$[A] = c_{Na_2A} + [Tl^+] - c_{TlClO_4} - \frac{l}{2} \tag{25}$$

[A] can be calculated from (25) as all quantities on the right-hand side are known. With the values of [A] thus obtained, $h_{0,2}$ was calculated according to (20). Table 2

shows that $h_{0,2}$ is constant with the mean value $h_{0,2} = (2.90 \pm 0.05)\,10^{-5}$. The constancy of the solubility product shows, that the assumption that all constants in the series $h_{1,1}$, $h_{3,1}$, $h_{5,1}$, *etc.* except $h_{1,1}$ are zero, upon which (25) is founded, was correct.

The distribution of the thallium in different ionic species in solutions saturated with Tl_2A can be obtained from Fig. 5 as a function of log [A] and from Fig. 6 as a function of log $[Tl^+]$. The $\bar{n}$-curves in Figs. 5 and 6 for the solutions which are saturated with respect to Tl_2A (s), have been calculated according to

$$\bar{n} = \frac{\Sigma G_y [Tl^+]^{-y}}{[Tl^+] + \Sigma H_y [Tl^+]^{-y}} = \frac{h_{1,1}[Tl^+]^{-1}}{[Tl^+] + h_{1,1}[Tl^+]^{-1}} \tag{26}$$

C. Solubility measurements on $NaTlA \cdot z\,H_2O$

As Tl_2A was the stable phase only for $[A] \leqslant 28$ mM the solubility investigations were extended to higher thiosulphate concentrations by the aid of $NaTlA \cdot z\,H_2O$, which is stable above this value. $NaTlA \cdot z\,H_2O$ was transferred into tubes containing solutions with the compositions of c_{Na_2A} M Na_2A, $(4 - 3\,c_{Na_2A})$ M $NaClO_4$ and the samples were then handled in the same way as described in B. The thallium content in the saturated solutions was determined as Tl_2CrO_4 and $[Tl^+]$ was evaluated from measurements in cell (1). The solid phase in equilibrium with the saturated solutions was identified as $NaTlA \cdot z\,H_2O$ by X-ray photographs (Guinier camera).

The dissolution of the salt can be represented in the following way:

$$m\,NaTlA \cdot z\,H_2O\,(s) + x\,A^{2-} \rightleftharpoons m\,Na^+ + Tl_m\,A_{m+x}^{(m+2x)-} + m\,z\,H_2O \tag{27}$$

The system is defined by the constants

$$k_{m,x} = \frac{[Na^+]^m\,[Tl_m\,A_{m+x}]}{[A]^x} \tag{28}$$

The solubility of the salt is then given by the equation

$$l_d = [Tl^+] + \sum_m \sum_x m\,k_{m,x}\,[A]^x\,[Na^+]^{-m} = [Tl^+] + \sum_x K_x\,[A]^x \tag{29}$$

where

$$K_x = k_{1,x}\,[Na^+]^{-1} + 2\,k_{2,x}\,[Na^+]^{-2} + 3\,k_{3,x}\,[Na^+]^{-3} + \cdots \tag{30}$$

(*cf.* Leden [12]).

The solubility product of the salt is

$$k_{1,-1} = [Na^+]\,[Tl^+]\,[A] \tag{31}$$

The results of the measurements are summarized in Table 3, where $[Na^+]$ has been calculated according to

$$[Na^+] = c_{NaClO_4} + 2\,c_{Na_2A} + l_d = 4 - c_{Na_2A} + l_d \tag{32}$$

For evaluation of the K_x constants in (29) l_d must be known as a function of [A] and therefore [A] was first calculated according to (5) with the constants

Table 3. The solubility of $NaTlA \cdot z\,H_2O$ (s) in sodium thiosulphate solutions at 25°C. Ionic strength 4 M. $K_0 = 0.006$ M, $K_1 = 0.006$, and $K_2 = 0.015\ M^{-1}$.

c_{Na_2A} mM	l_d mM	$[Tl^+]$ mM (2)	[A] mM	$[Na^+]$ M (32)	$k_{1,-1}$ M^3 10^3 (31)
14	30.35	23.7	37.5	4.02	3.57
24	26.46	20.0	44.3	4.01	3.55
34	24.16	17.9	51.0	4.00	3.60
54	20.27	13.9	66.3	3.97	3.65
84	16.75	10.2	92.0	3.94	3.68
150	13.51	6.35	154	3.87	3.79
250	12.73	4.27	251	3.76	4.02
300	12.91	3.66	296	3.71	4.01
375	13.22	3.11	371	3.64	4.18
450	14.27	2.75	443	3.56	4.34
550	16.33	2.44	552	3.47	4.68
550	16.05	2.39	552	3.47	4.55
600	17.15	2.29	598	3.43	4.68
650	18.36	2.21	640	3.37	4.78
700	19.29	2.11	700	3.32	4.91
800	21.90	2.00	795	3.22	5.12
900	25.04	1.94	893	3.13	5.42
1000	29.18	1.86	990	3.03	5.57
1100	33.51	1.83	1080	2.93	5.80
1200	38.86	1.80	1180	2.84	6.02
1333	47.32		1280	2.72	

evaluated from the potentiometric measurements (see Table 4). Preliminary K_x constants were then evaluated by linear extrapolation and with these constants $\bar{n}$ was calculated according to

$$\bar{n} = \frac{\Sigma\,(m+x)\,k_{m,x}\,[Na^+]^{-m}\,[A]^x}{[Tl^+] + \Sigma\,K_x\,[A]^x} \tag{33}$$

under the assumption that $K_0 = k_{1,0} \cdot [Na^+]^{-1}$, $K_1 = k_{1,1} \cdot [Na^+]^{-1}$, and $K_2 = k_{1,2} \cdot [Na^+]^{-1} + 2\,k_{2,2}\,[Na^+]^{-2}$. $\bar{n}$ was represented in a diagram as a function of [A] and in this diagram straight lines were drawn according to (7) with the slope $-l_d^{-1}$ intersecting the abscissa at c_A. From the points of intersection with the curve, the corresponding values of [A] could be read off (*cf.* Leden and Nilsson [13]). New values of K_x were then evaluated by linear extrapolation. $K_0 = 0.006$ was obtained as the origo ordinate in a $l_d - [Tl^+]$ *vs.* [A] diagram. The experimental points in a $(l_d - [Tl^+] - K_0)\,[A]^{-1}$ *vs.* [A] diagram could be represented by the linear relationship $K_1 + K_2\,[A] = 0.006 + 0.015\,[A]$, thus giving the constants K_1 and K_2.

The $\beta_{m,n}$ constants can be recalculated to $k_{m,x}$ according to the equation

$$\frac{k_{m,x}}{[Na^+]^m} = \beta_{m,n} \left(\frac{k_{1,-1}}{[Na^+]}\right)^m = \beta_{m,n}\,([Tl^+]\,[A])^m \tag{34}$$

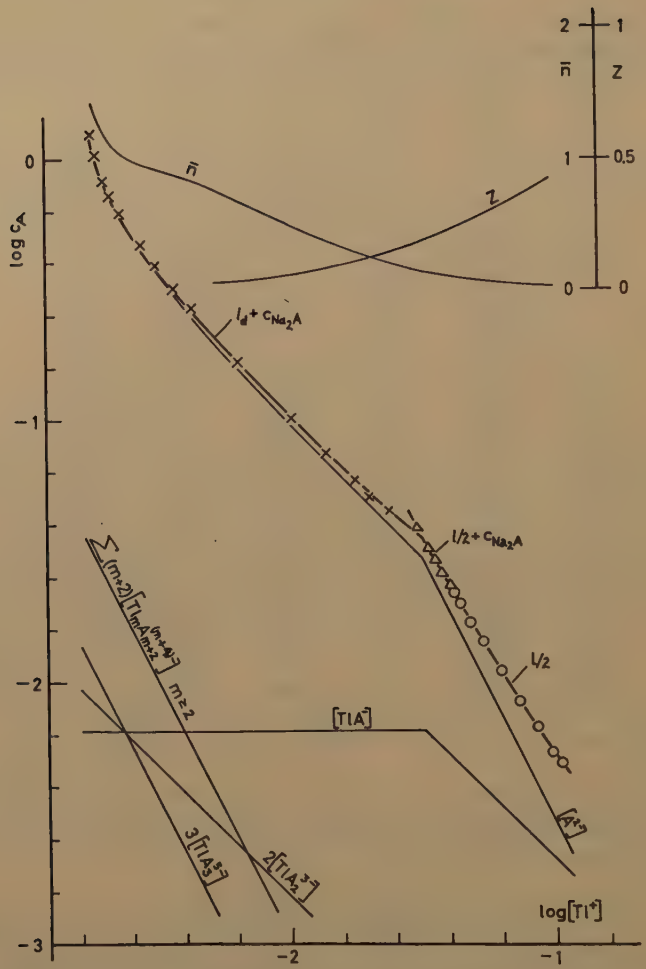

Fig. 6. The logarithm of the total thiosulphate concentration, Z (defined by equation (35)), and the ligand number $\bar{n}$ as functions of the logarithm of the free thallium ion concentration. Symbols as in Fig. 5. The concentration of a complex at a certain value of log $[Tl^+]$ can be read off in a logarithmic scale from the line marked with the symbol of the complex ion in question. The slopes of the lines are $m-n$ in the left-hand part of the diagram, where the solutions are saturated with $NaTlA \cdot z\,H_2O$ (s) and $m-2n$ in the right-hand part, where the solutions are saturated with Tl_2A (s).

In these calculations the value of $[Tl^+][A]$ was used which was valid in the range where the complex in question dominated. $\beta_{1,1}$ thus gives $\frac{k_{1,0}}{[Na^+]} = 0.007$ which is larger than the value $K_0 = 0.006$ evaluated from the solubility measurements. Thus $k_{m,0}\,(m>1)$ seem to be zero. $\beta_{1,2}$ according to (34) gives $\frac{k_{1,1}}{[Na^+]} = 0.007$ which is larger than the evaluated value $K_1 = 0.006$ and this shows that $k_{m,1} = 0$ $(m>1)$.

Table 4. Equilibria constants used to describe the measurements in this paper. Temperature 25°C. Ionic strength 4 M.

Method	$\beta_{1,1}$	$\beta_{1,2}$	$\beta_{1,3}$	$h_{0,2}$	$h_{1,1}$	$k_{1,-1}$	$\frac{k_{1,0}}{[Na^+]}$	$\frac{k_{1,1}}{[Na^+]}$	K_2	Polynuclear complexes
	M^{-1}	M^{-2}	M^{-3}	M^3	M^2	M^3	M		M^{-1}	
potentiometric trations	7.2	5.2	1.5		$2.1 \cdot 10^{-4}$ [1]		0.007[2]	0.007[3]	0.007[4]	Tl_2A_4; $\beta_{2,4} = 350$
sol. measurements n Tl_2A(s)				$2.9_0 \cdot 10^{-5}$	$2.1 \cdot 10^{-4}$					
sol. measurements n $NaTlA \cdot zH_2O$ (s)						$3.6 \cdot 10^{-3}$ [5]	0.006	0.006	0.015	Tl_mA_{m+2}

[1] calculated from $\beta_{1,1}$ according to (21)
[2] calculated from $\beta_{1,1}$ according to (34)
[3] calculated from $\beta_{1,2}$ according to (34)
[4] calculated from $\beta_{1,3}$ and $\beta_{2,4}$ according to (30) and (34)
[5] at low values of c_A

$\beta_{1,3}$ gives $\frac{k_{1,2}}{[Na^+]} = 0.003$ and $2\beta_{2,4}$ gives $\frac{2k_{2,2}}{[Na^+]^2} = 0.004$. Thus $K_{2(calc.)} = 0.003 + 0.004 = 0.007$, which is only about half the value $K_2 = 0.015$ which was evaluated from the solubility measurements. This leaves the possibility open that $k_{m,2} > 0$ $(m > 2)$.

The point where Tl_2A is transformed to $NaTlA \cdot zH_2O$ is given by $[A] = (k_{1,-1} \cdot [Na^+]^{-1})^2 h_{0,2}^{-1} = 0.028$ M.

The distribution of the thallium in different complexes in saturated solutions can be read off from the straight lines in Figs. 5 and 6.

D. Discussion

The constants determined in this paper have been collected in Table 4 where also the β constants have been recalculated to $h_{m,y}$ and $k_{m,x}$ constants. The agreement between the results obtained by different methods is acceptable except in the case of the value of K_2. The spreading of the F-curves belonging to different c_{Tl} in Figs. 1 and 2 could be approximately explained by the complex Tl_2A_4 with $\beta_{2,4} \approx 350$ which together with $\beta_{1,3} = 1.5$ gives $K_2 = 0.007$ while from the solubility measurements, $K_2 = 0.015$ was evaluated. It must be remembered that $\beta_{2,4}$ and K_2 were evaluated at high thiosulphate concentrations and the existence of polynuclear complexes must therefore be considered as uncertain, as the activity factors change when c_A is raised. This is shown by the variation of $k_{1,-1}$ in Table 3. (Part of the variation must be accredited to varying diffusion potentials.) The results obtained by the two methods, however, agree qualitatively, in that all polynuclear complexes belong to the same series $Tl_m A_{m+2}$ and this speaks in favour of their existence. It is not possible to explain the inconsistency of $k_{1,-1}$ by assuming the presence of sodium thiosulphate complexes. Denney and Monk [14], and Gimblett and Monk [15] found $\beta_{NaA} - \approx 4$ at the ionic strength of zero, but it is possible that this constant is rendered smaller at the ionic strength used during the present investigation (*cf.* Nilsson [16], discussion). The disagreement between the potentiometric and the

solubility measurements is shown in Fig. 5, where the dotted curve has been calculated with the β-constants, while the full drawn curve has been drawn by the aid of the K_x-constants.

The distribution of thallium as a function of log [A] is found in Fig. 3 for two thallium contents and in Fig. 5 for saturated solutions. It is obvious that the ion TlA^- is the dominating complex when $[A] < 1$ M. At this value the concentration of the next complex, TlA_2^{3-}, approximately equals the concentration of TlA^-. As the free thallium ions at low thiosulphate concentrations make up the main part of c_{Tl}, the total thiosulphate concentration in Fig. 6 was drawn as a function of log $[Tl^+]$. The right-hand part of this figure shows how the dissociation of the TlA^- ion is halted when c_{TlClO_4} is increased in the thallium perchlorate solutions, saturated with Tl_2A (s). This is also illustrated by the rise of the curve

$$Z = \frac{c_{Tl} - [Tl^+]}{c_A} \qquad (35)$$

List of symbols

$[A]$	$= [S_2O_3^{2-}]$, free thiosulphate concentration
c_{Na_2A}	sodium thiosulphate added
c_A	total concentration of thiosulphate
c_{Tl}	total thallium content
$\bar{n}$	ligand number (7), (26), (33)
Z	number of thallium atoms bound per thiosulphate group (35)
E_m	emf (in mM) of cell (1)
E	emf (in mV), defined by (3)
$\beta_{m,n}$	complexity constant (4)
F	$= c_{Tl}/[Tl^+]$
F_1	$= (F-1)/[A]$ (9)
F_2	$= (F_1 - \beta_{1,1})/[A]$ (10)
$\alpha_{m,n}$	defined by (12)
$l/2$	solubility of Tl_2A
l_d	solubility of $NaTlA \cdot z\,H_2O$ (29)
$h_{m,y}$	equilibrium constant (15)
$h_{0,2}$	solubility product of Tl_2A (20)
H_y	sum of equilibrium constants (18), (19)
G_y	sum of equilibrium constants (23), (24)
$k_{m,x}$	equilibrium constants (28)
$k_{1,-1}$	solubility product of $NaTlA \cdot z\,H_2O$ (31)
K_x	sum of equilibrium constants (30)

SUMMARY

The complex formation between thallium(I) and thiosulphate ions in water solutions was studied at 25°C and at an ionic strength 4 M with sodium perchlorate as an additional salt. Three different investigations were made.

A) Potentiometric titrations at which $[Tl^+]$ was measured as a function of the total thiosulphate concentration with the total thallium content kept constant within each series.

B) Solubility determinations on $Tl_2S_2O_3$ (s) in sodium thiosulphate and thallium perchlorate solutions.

C) Solubility measurements on $NaTlS_2O_3 \cdot z\,H_2O$ (s) in sodium thiosulphate solutions.

The following complexes were found to exist, $TlS_2O_3^-$, $Tl(S_2O_3)_2^{3-}$, $Tl(S_2O_3)_3^{5-}$, and $Tl_m(S_2O_3)_{m+2}^{(m+4)-}$. (As for the polynuclear complexes, see discussion.) The evaluated constants are collected in Table 4.

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Purification of antihemophilic globulin

I. Some studies on the stability of the antihemophilic globulin activity in fraction I-0 and a method for its partial separation from fibrinogen

By Margareta Blombäck

The observation made by Addis in 1911, that a globulin fraction prepared from normal human plasma corrected the coagulation time of hemophilic blood *in vitro*, was confirmed by Bendien & van Creveld in 1935 and 1936, and independently by Patek & Taylor in 1936 and 1937.

In 1944–1946, the fractions of Cohn's method 6 were assayed for antihemophilic globulin activity by the Thorndike laboratory group (see Edsall *et al.*, 1944; Taylor *et al.*, 1945; Minot *et al.*, 1945; Lewis *et al.*, 1946) and the activity found to reside mainly in fraction I. The commercial preparations of fraction I have not, however, been used with great success *in vivo* (Aggeler, 1956; Alexander, 1956; Rosenthal, 1956; Koller, 1955; Winterstein, 1955; Deutsch, 1955; Achenbach, 1957). Tested *in vitro* with more sensitive methods than those initially used, the activity did not reach more than a small percentage of that of the original plasma (Alexander & Landwehr, 1948; Spaet & Kinsell, 1953; Winterstein, Marbet & Strässle, 1956; Brinkhous, Penick, Langdell, Wagner & Graham, 1956). Freshly prepared fraction I, however, often proved to be more active (Winterstein, 1955; Egli & Kesseler, 1956; Hörder, 1957). As pointed out already by the Thorndike group, contaminating prothrombin as well as plasminogen and its proactivator made the product instable and, furthermore, difficulties arose in sterilizing the fraction. In 1946 van Creveld & Mastenbroek analyzed the fibrinogen fraction of Kekwick, Mackay & Record (1946) for antihemophilic activity, and found a normalizing effect on the coagulation time *in vivo*.

The results of investigations on AHG preparations made before about 1950 are difficult to evaluate, since the methods generally used for assay (coagulation time) were fairly insensitive and some of the negative results might have been due to the fact that the patient was suffering from hemophilia B.

During the past five years, methods for purification of antihemophilic globulin have been elaborated by several authors, using different starting material and different ways of preparation. Some started with fractions earlier known to contain antihemophilic activity, such as the globulin fraction of Patek & Taylor, the fibrinogen fraction of Kekwick *et al.* or of Cohn *et al.*, whereas others started from plasma (Bidwell, 1955; van Creveld, Hoorweg, Den Ottolander & Veder, 1956; Kekwick & Wolf, 1957; Shinowara, 1956; Spaet & Kinsell, 1953; Surgenor & Steele, 1957; Wagner, Pate & Brinkhous, 1954; Wagner, McAllister & Brinkhous, 1956; Wagner & Thelin, 1956; Wagner, Richardson & Brinkhous, 1957).

In 1956, a method for purification of fibrinogen starting with Cohn's fraction I was elaborated in this laboratory (Blombäck & Blombäck). Freshly prepared fraction I was extracted with an ethanol-citrate solution containing glycine in a concentration of 1 *M*. At this glycine concentration the fibrinogen is nearly insoluble, whereas the contaminating proteins, such as prothrombin, have a high solubility. After extraction, the antihemophilic globulin (AHG) remained in the fibrinogen fraction (I-0), which was stable in solution. Its use in various hemorrhagic conditions has been described by Nilsson, Blombäck, Blombäck & Svennerud (1956), Nilsson, Blombäck & von Francken (1957), Nilsson, Blombäck, Jorpes, Blombäck & Johansson (1957) and Blombäck & Nilsson (1958). Although the yield of AHG was usually very good as judged from the results *in vitro* and *in vivo*, there was some variability which was difficult to explain. Further studies have to some extent clarified this discrepancy, and shown it to be due chiefly to the following factors:

1. The somewhat low stability of AHG at the pH at which the extractions were performed (pH 6.0).
2. Incipient clotting during collection of the blood.
3. The temperature and rate at which the AHG solutions were frozen down.

In the present paper, an account is given of a modified procedure for the preparation of fraction I-0, with a more constant yield of AHG. In addition, a method is described for separation of the antihemophilic globulin from the fibrinogen in the modified fraction I-0.

Methods

Total protein and fibrinogen concentrations were determined spectrophotometrically according to Blombäck & Blombäck (1956) and Blombäck (1958). The *coagulability*, i.e., the ratio of protein coagulable with thrombin to total protein in the different fractions, was calculated as described by these authors.

Antihemophilic globulin activity.—This was investigated with the technique of Nilsson, Blombäck & von Francken (1957) in at least two dilutions prepared in saline; i.e., on hemophilic A plasma in a recalcification system in which the ability of the preparations to correct the prolonged recalcification time of the hemophilic plasma was measured. A freeze-dried preparation of fraction I-0 (or the original plasma) was used as a standard. It was also checked that the activity was not due to thromboplastin activity, by investigating whether addition of the preparation to hemophilic B or normal plasma shortened the recalcification time.

Procedure for preparation of the modified human fraction I-0

Since heavy losses had been found to occur in sterile filtration, the whole preparation procedure was carried out in a closed system and under sterile conditions, when the fraction was intended to be used *in vivo* (cf. Kekwick & Mackay, 1954; Kekwick & Wolf, 1957).

Collection of blood.—Only blood from donors in whom venipuncture had been performed without any complications was used. The blood was allowed to run during magnetic stirring into a solution of trisodium citrate (one part 4.0 % $Na_3C_6H_5O_7 \times 2\ H_2O$ to 7 parts of blood) in siliconized centrifuge bottles (fitting the International Refrigerated Centrifuge Model SR-3). Immediate centrifugation at 2000 *g* and +4°C for 30 minutes. The plasma was siphoned off and centrifuged again for 20 minutes.

Fraction I was precipitated according to the method of Cohn *et al.* (1946). Centrifugation for 25 minutes at 2000 *g*, −3°C.

Extraction 1 (*E-1*).—250 ml of a 0.055 *M* trisodium citrate solution, containing 6.5 % ethanol and glycine to a concentration of 1 *M*, and adjusted to pH 6.80 ± 0.05, was used per litre of original plasma. The chilled (−2 to −3°C) solution was slowly added to fraction I under cautious stirring, until a homogeneous paste was obtained. The rest of the solution was added and the suspension stirred for one hour at −2 to −3°C. Centrifugation was carried out at 2000 *g* for 10–12 minutes at the same temperature.

Extraction 2 (*E-2*) was performed with a fresh volume of the extraction solution and under the same conditions as extraction 1. Centrifugation for 15 minutes.

I-0.—The non-extractable residue, the protein of which contained about 85 % fibrinogen, was dissolved in citrate buffer (0.055 *M* sodium citrate solution, pH 6.80 ± 0.05), about one-tenth or more of the original plasma volume. The first portion of the solution added was chilled. The vessel was then transferred to a water bath of +30°C and the fraction dissolved as rapidly as possible. The final volume was about one-eighth or somewhat more of the original plasma.

The preparation was then immediately shell-frozen at −30 to −50°C, and freeze-dried, or rapidly frozen at the same temperature. All samples for assay were rapidly frozen at −30 to −50°C. Both the freeze-dried preparations and the frozen solutions were stored at −23 to −30°C. The sterile preparations were then dissolved in the same volume of 0.05 *M* NaCl as before freeze-drying, in order to make an isoosmolar solution.

Preparative procedure for partial separation of AHG and fibrinogen in human fraction I-0

I-1-A.—Fraction I-0 was dissolved as described above to about 1.6 % of protein, and further diluted with the citrate solution to a protein concentration of 0.75 % and chilled to 0°C. Two volumes of a 0.45 *M* glycine solution (0°C) were added. Ethanol (10 % v/v), 0°C, was added dropwise until a concentration of 0.5 % was reached. Gentle stirring for 15 minutes. A precipitate (I-1-A) containing the antihemophilic activity was obtained. It was collected by centrifugation for 10 minutes at ±0°C, 2000 *g*.

This precipitate, as well as that eventually assembled on the glass rod (the latter, if present, being kept in citrate solution at 0°C while the main part was centrifuged), was dissolved in 0.055 *M* citrate solution, pH 6.80 ± 0.05, in about the same volume as fraction I-0. The precipitate was first cautiously broken up at ±0°C; the vessel was then transferred to a water bath of +30°C and the precipitate dissolved as rapidly as possible.

I-2-F.—The supernatant of fraction I-1-A was chilled to −3°C, and ethanol (10 % v/v) (−3°C) added dropwise to a concentration of 6.5 %. Centrifugation, −3°C, 10 minutes, 2000 *g*. The precipitate (I-2-F) containing the fibrinogen was dissolved in 0.055 *M* citrate solution, pH 6.80 ± 0.05, the first part of the citrate solution being chilled and the vessel later transferred to a water bath of +30°C.

The two fractions, I-1-A and I-2-F, were rapidly frozen at −30 to −50°C, and if possible, freeze-dried immediately. The fractions and the samples for assay were then stored at −23 to −30°C. Whenever possible, the latter were assayed immediately, i.e., before freezing down. Usually, however, they were stored for 1–10 days, owing to shortage of hemophilic plasma for testing.

Experimental

Studies on factors influencing the yield of AHG

As mentioned above, the activity of fraction I-0 was found to be variable. Some of the conditions for preparation of the original fraction I-0 (Blombäck & Blombäck, 1956) were therefore investigated more thoroughly.

1. *Collection of the blood.*—Although siliconized bottles were used, small clots were sometimes detected when the blood cell suspension was filtered through gauze. The yield of antihemophilic activity in fraction I-0 was always low on these occasions. The citrate concentration was therefore slightly raised (1 part 4.0 % trisodium citrate solution to 7 parts of blood), but better results were obtained only after magnetic stirring during the collection of the blood was introduced (the bottles were previously shaken by hand).

2. *pH optimum.*—Since the quantitative results were still variable, the pH optimum for extraction giving the best yield of antihemophilic activity in fraction I-0 was investigated, starting with 150–200 ml of plasma in each batch. The yield of antihemophilic activity in fraction I-0 was studied after extraction of fraction I twice at pH 6.0, 6.3, 6.8 and 7.3, i.e., the citrate–glycine–ethanol mixture was adjusted to these pH values. The results of two experiments can be seen in Tables 1 A, B and C. The optimal values in this respect proved to be pH 6.8 and 7.3.

Thus, it can be seen not only that the solubility of the antihemophilic activity in the extraction mixture was greater at pH 6.0, but also that the instability of this activity was greatest at this pH. Furthermore, it can be concluded that pH 6.8 and 7.3 seem to be equally good with respect both to the insolubility of the antihemophilic activity and to its stability.

3. *Freezing conditions.*—Some of the preparations were more active *in vivo* than *in vitro*. The preparations for clinical use had been rapidly frozen down, whereas the samples for testing *in vitro* were put in an ice-box (about −10 to −20°C). The influence of freezing on the antihemophilic activity was therefore investigated.

When samples of fraction I-0 (citrate solution pH 6.3) had been rapidly frozen down at temperatures between −25 and −70°C, no significant difference was found between their activity. Nor was any difference noted between the activity of samples frozen rapidly or slowly at −25°C. If, however, they were frozen down at e.g. −10 or −15°C, the samples frozen slowly proved to have lost 50–75 % of their activity and, when kept at −5°C, almost all the activity was lost in the course of a few hours, in comparison to that of rapidly frozen samples. It can also be mentioned that samples frozen and then thawed immediately without previous freeze-drying may show a decrease in activity of about 30 % as compared to that of fresh samples.

An investigation of the antihemophilic activity at temperatures stated to be critical was also made. Since some authors, e.g. van Creveld *et al.* (1956), have purified antihemophilic activity by precipitating the fibrinogen at 56°C, it was thought of interest to investigate this possibility. When heating fraction I-0 at 56°C (in citrate solution pH 6.3–6.4) for 2–15 minutes and testing the supernatant of the precipitated fibrinogen for activity, only about 20 % of the antihemophilic activity was left. Further experiments showed that no activity was lost at 45°C for 5 minutes; 50–60 % was lost at 50°C for 5 minutes, and some activity (30 %) could be eluted from the small amount of fibrin precipitated at this temperature. At 54°C

Table 1 A. Yield of antihemophilic activity, protein and fibrinogen in per cent of total (E-1 + E-2 + I-0), when fraction I is extracted at different pH values.

	AHG activity % of total								Fibrinogen content % of total								Protein content % of total							
pH...	6.0		6.3		6.8		7.3		6.0		6.3		6.8		7.3		6.0		6.3		6.8		7.3	
Exp....	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E-1	38	29	15	—	3	7	—	7	3	4	4	—	3	4	—	4	31	37	35	—	36	38	—	36
E-2	5	28	3	—	1	5	—	5	0.3	0.2	1	—	1	1	—	0.5	4	5	5	—	7	7	—	6
I-0	57	44	82	—	96	89	—	88	97	96	96	—	97	95	—	96	65	58	60	—	57	55	—	58
Coagulability, %:									83	85	86	83	89	87	—	87								

Table 1 B. Yield of antihemophilic activity in per cent of that of normal human plasma.

In experiment 1 the samples were tested immediately, in experiment 2 they had been frozen down and preserved at −25°C for one day.

pH...	6.0		6.3		6.8		7.3	
Exp....	1	2	1	2	1	2	1	2
E-1	13	21	6	9	3	5	—	5
E-2	2	21	1	—	1	4	—	4
I-0	19	31	35	48	92	68	—	63

Table 1 C. AHG activity per mg protein in fraction I-0 (exp. 2).

If plasma is arbitrarily assumed to contain 100 units AHG per ml, its activity will be 1.43 units per mg protein.

pH...	6.0	6.3	6.8	7.3
Units/mg protein	14.5	24.8	34.8	32.4
Purification	10.2	17.6	24.3	22.7

for 5 minutes, the residual activity in the supernatant was only 10 % (*cf.* Bidwell, 1955). No activity could be eluted from the fibrin. About two minutes elapsed before the desired temperature was reached.

Studies of conditions for separating fibrinogen from AHG in fraction I-0

When the purification technique for bovine and human fibrinogen was elaborated (Blombäck & Blombäck, 1956), the various fractions were assayed by I. M. Nilsson and the present author for their antihemophilic activity. It was observed that fraction I-0, bovine or human, contained the AHG of the plasma in good yield, and

Table 2. Separation of AHG activity from fibrinogen of fraction I-0, expressed in relative amounts.

	pH 6.3			pH 6.8		
	AHG activity	Fibrinogen content	Coagulability %	AHG activity	Fibrinogen content	Coagulability %
I-0	100	100	87	100	100	84
I-1-A	95	25	75	80	13	66
I-2-F	4	61	90	13	74	95
Supernatant I-2-F	6	2	—	5	3	—

that on further purification of both bovine and human fibrinogen, the activity disappeared in some way or other, or at any rate could not be recovered in any yield. When, however, an ionic strength of 0.1 (ethanol 0.75 %, glycine 0.5 *M*, protein concentration 0.6 %), instead of 0.3 was applied in precipitating fraction I-1 from the dissolved bovine fraction I-0, a precipitate, I-1-A, was obtained which contained about the same antihemophilic acitivity as did fraction I-0 itself, most of the fibrinogen remaining in the supernatant.

This observation called for an investigation of the effect of variations in ionic strength, ethanol and glycine concentration on the separation of AHG from fibrinogen in fraction I-0. All the first experiments were made on bovine fraction I-0.

Ethanol concentration.—Ethanol concentrations of 0.4, 0.75 and 1.5 % were tried (±0°C, protein concentration 0.3 %, glycine 0.5 *M*, pH 6.35, ionic strength 0.1). A concentration of 1.5 % gave the best yield in fraction I-1-A, but also the highest protein (fibrinogen) concentration. About equal amounts of protein appeared in precipitate and supernatant with concentrations of 0.4 and 0.75 %. 0.5–1 % were the concentrations subsequently used.

Ionic strength.—Tests were made with ionic strengths of 0.15, 0.1, 0.075 and 0.055. Ionic strength 0.1 gave the best yield. 77 per cent of the total antihemophilic activity was recovered in fraction I-1-A, while 60 % of the protein remained in the supernatant.

Glycine concentration.—Concentrations of 0.25, 0.50, 0.75 and 0.95 *M* were tested. The best separation was achieved with a concentration between 0.25 and 0.50 *M*.

On the basis of these observations, the procedure was applied to human material, and the results were equally good. Ionic strength 0.1, protein concentration 0.25 %, 0.3 *M* glycine, pH 6.35, 0.5 % ethanol were the conditions originally used.

Since, however, as mentioned above, the optimum pH for stability of the activity was found to be pH 6.8, the possibility of performing precipitation at this pH was investigated. It can be inferred from Table 2 that there was no appreciable discrepancy between the results obtained at these two pH values.

In three experiments performed at pH 6.8, the purification in fraction I-1-A per mg protein compared with fraction I-0 was 4.4, 4.4 and 4.9.

After precipitation of fraction I-1-A, the fibrinogen could be precipitated from the supernatant at an ethanol concentration of 6.5 %.

Discussion

The stability, or rather instability, of antihemophilic globulin (AHG) has been a problem encountered by most clinicians since the introduction of blood or blood derivatives (plasma, serum and plasma fractions) in the treatment of hemophiliacs. Sköld (1944) stressed that bank blood was less effective than fresh blood, and that when stored 1–2 months, it did not shorten the coagulation time. Plasma obtained by immediate centrifugation after withdrawing the blood, as well as fresh dried plasma, seemed to have the same coagulation-promoting effect as fresh blood, when judged by the influence on coagulation time. The results obtained before 1950–1952 are sometimes difficult to evaluate, since the existence of hemophilia B was not known, or at any rate not accepted, until this time. Moreover, the methods generally used earlier for studying the stability of AHG were somewhat insensitive. A review of earlier known data and a thorough study of the stability of AHG in blood and plasma were given in 1956 by Penick & Brinkhous. They, as well as Alexander *et al.* (1954), pointed out that if any thrombin is formed during collection of the blood, it causes a loss of AHG (cf. Rizza & Walker, 1957). Careful collection of the blood and prompt mixing with the anticoagulant are recommended by them. It can be recalled that, in the present experiments, good yields were more constantly seen in fraction I-0 when the blood was collected during magnetic stirring.

The stability of AHG in plasma at different temperatures (+56 to −20°C) was also studied by Penick & Brinkhous (1956). They found that the lower the temperature, the better was the preservation of AHG; thus, 60–90 % seemed to remain after storage for one week at −20°C.

It can be inferred from the present experiments that the speed of freezing is also of importance. Thus, rapid freezing of fraction I-0 or fraction I-1-A at about −30°C (in citrate solution at pH 6.3) gave the most constant values. Slow freezing at, e.g. −15°C, produces a great loss of activity, and at −5°C almost all activity is lost in a short time.

The instability at higher temperatures of the antihemophilic activity in fraction I-0 seems to agree fairly well with the findings of Bidwell (1955). She also pointed out that the pH optimum for the stability of her bovine AHG preparation is about pH 6.8. At this pH, her fraction could be heated at +48 to +49°C (for five minutes) without loss of activity, whereas at pH 6.0 and 7.6, 50 % and 15 %, respectively, were lost. As can be concluded from the experiments in Tables 1 A and B, not only the solubility, but also the stability of the antihemophilic activity during the extractions is influenced by pH. The pH optimum is in good agreement with that found by Bidwell.

During the past five years, several methods have been described for the purification of antihemophilic globulin. Since the crude antihemophilic globulin preparations used as starting material contain fibrinogen as well, some workers have also tried to separate the antihemophilic activity from the fibrinogen.

Bidwell succeeded in preparing a bovine fraction 100–400 times as active as the original plasma per mg N. These preparations, made by salt precipitation of $Al(OH)_3$ adsorbed plasma, were also used with good effect *in vivo*. Their use is, however, limited by their antigenicity.

In some methods for purification, use is made of the possibility of removing the fibrinogen by heating at 56°C, a method used for plasma by Lozner, Kark & Taylor (1939). Starting with fraction I or the fraction of Kekwick *et al.* (1946), van

Creveld *et al.* claimed that they had, in this way, obtained a 20 times greater concentration of AHG than that of plasma, the activity being about 28 times that of plasma per mg protein. The data given by these authors are, however, inconclusive, and great losses were observed during heating. Spaet & Kinsell used bovine fraction I heated at 56°C. After further adsorption of impurities on $BaSO_4$, and precipitation at pH 5.4, a product was obtained 70 times more active than fresh human plasma. Bidwell observed great losses of AHG, (90 % or more), when heating AHG fractions at 56°C for 5 minutes. This also applied when fraction I-0 was heated at this temperature.

Procedures for purifying the antihemophilic activity by adsorption have been devised. Bidwell investigated the method of Lorand & Laki (1954) who tried adsorption on kaolin. However, she found only a slight Christmas factor effect in the eluate. Adsorption on kaolin was also used by van Creveld *et al.* (1956) starting with the purified fraction mentioned above.

A promising technique for the purification of the antihemophilic factor was devised by Wagner *et al.* (1954, 1956, 1957). They were able to prepare antihemophilic globulin fractions (canine) by selectively adsorbing fibrinogen on fuller's earth from $BaSO_4$ adsorbed plasma and further purification by ethanol or salt precipitation. A product 200 times purified per mg of protein was obtained.

In 1957 Kekwick & Wolf, by a modification of Kekwick's procedure for plasma fractionation with ether, obtained a fibrinogen fraction containing AHG in good yield with an activity of 20–25 times that of the original plasma per mg protein. This preparation was used *in vivo* with good results.

Fraction I was used by Shinowara who, according to a preliminary report (1956), precipitated the fibrinogen with ether; 77 % of the TPC (thromboplastic plasma component) or "antihemophilic substance" remained in the solution, contaminated with less than 3 % of fibrinogen. This fraction was further purified at least twice by ether and ethanol fractionation in the presence of glycine. Shinowara stated that about 2 ml of an isotonic 4 % solution of the fraction is equivalent to the TPC activity in 300 ml of citrated plasma, assayed on hemophilic whole blood. No mention is made of its activity *in vivo*.

In 1957, Surgenor & Steele devised a method for separating the antihemophilic globulin activity from fibrinogen in human fraction I by dissolving this fraction in a solution containing dextran sulphate of high molecular weight, whereby the AHG but not the fibrinogen was dissolved. The AHG was further purified by dialysis. The activity could be destroyed at 56°C for 20 minutes; it was relatively stable at pH 5.5–7.0. However, the fibrinogen could not be recovered after the treatment with dextran sulphate. No mention was made of its effect *in vivo*.

With the method described in the present paper, it is possible to separate partially the fibrinogen from the antihemophilic globulin activity in fraction I-0 at low ionic strength in the presence of glycine, with a good yield of both substances. 75–95 % of the AHG activity of fraction I-0, 4.5 times purified, is recovered in the precipitate I-1-A, whereas the fibrinogen remains in the supernatant, and can be precipitated with a yield of 60–80 % of that in fraction I-0, and with a coagulability of 90–95 %. The purification of the AHG activity was about 100 times that of plasma per mg protein. Glycine was originally introduced in this step, since it was supposed that it might help in dissociation of the protein complexes in fraction I-0. However, it may also function as a protective reagent for the antihemophilic

activity, as seems to be the case when the parathormone B is subjected to electrophoresis in the presence of glycine (Rasmussen, 1957).

In view of the fact that the isolated antihemophilic globulin fraction has not yet been tested for its activity *in vivo*, this report is to be considered as preliminary.

SUMMARY

The yield of antihemophilic globulin (AHG) activity in fraction I-0, the purified Cohn's fraction I, has been studied. It has been found that if extraction with glycine is performed at pH 6.8, instead of at pH 6.0 as originally devised, good yields are obtained. The influence of different temperatures (-70 to $+56$°C) on the activity has been studied.

A method is described for the separation of antihemophilic globulin activity from the fibrinogen in fraction I-0. The AHG activity can be precipitated at low ionic strength in the presence of glycine. The yield will be about 80 per cent and the purification is 100 times that of plasma calculated per mg protein. The fibrinogen can be recovered from the supernatant by precipitation. The yield will be about 70 per cent of the amount present in fraction I-0, the coagulability being about 92 per cent.

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